TISSUE COPPER ACCUMULATION AS A MEASURE OF BIOAVAILABILITY IN CHICKS AND SHEEP

BY

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TO

ZENA,

RICHARD

AND

SHANE

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Six experiments were conducted to investigate the use of tissue Cu accumulation during short term, high level Cu supplementation as bioassay criterion to determine Cu availability from inorganic sources fed to chicks and sheep. In Experiment 1, chicks were fed diets supplemented with up to 300 mg/kg Cu from reagent grade (RG) cupric acetate for 22 days to determine the relationship between dietary Cu and tissue Cu. Copper concentrations in plasma and liver increased with increasing dietary Cu. In Experiment 2, chicks were fed the same levels and source of Cu as in Experiment 1 for 1, 2 or 3 weeks to determine the effect of age on tissue Cu accumulation. Liver was the only tissue sensitive to Cu at weeks 2 and 3. Experiment 3 was a 3-week bioassay conducted to determine Cu availability from RG acetate, feed grade (FG) oxide, FG sulfate and FG carbonate.

Acetate and sulfate were equally available and were both more available than carbonate which, in turn, was more available than oxide. The relationship between dietary Cu and tissue Cu in sheep fed up to 45 mg/kg added Cu from RG acetate for 15 or 30 days was investigated in Experiment 4. Copper concentration in livers of sheep fed added Cu was not affected by added dietary Cu, but was higher than that of controls and tended to increase with time. In Experiment 5 sheep depleted of Cu for 3 weeks were supplemented with up to 80 mg/kg Cu from RG acetate for 10 or 20 days. Liver was the only tissue sampled in which Cu accumulated and the 10-day assay was optimum. Experiment 6 was a bioavailability assay. Sheep were depleted for 3 weeks, liver biopsied to determine initial Cu concentration and then supplemented for 10 days with the same Cu sources as in Experiment 3. The assay was incapable of differentiating among sources evaluated; however, surgery trauma during biopsy may have confounded the results. These studies indicate that tissue Cu accumulation can be used as a bioassay criterion for determining Cu bioavailability in chicks. Results of sheep studies, however, were inconclusive.

CHAPTER I
INTRODUCTION

Copper (Cu), an essential element for animals and plants, is a member of group 1B of the periodic table of elements and consists of two natural isotopes: ^{63}Cu and ^{65}Cu (69.1 and 30.9%, respectively; NRC, 1977). The element forms more than 200 minerals but occurs primarily as copper sulfide deposits in igneous rocks (Georgievskii, 1982). Its concentration in the earth's crust has been estimated at 50 mg/kg, with soil content ranging from 1 to 50 mg/kg with an average of approximately 20 mg/kg (NRC, 1977).

The presence of Cu in living matter was first demonstrated in the early 1800s when it was shown to be present in both plant and animal tissues (Miller et al., 1979). Copper concentrations in plant material have since been shown to range from as low as 1 mg/kg to as much as 50 mg/kg on a dry tissue basis (NRC, 1977). Animal tissues also show a wide range of Cu concentrations; however, Cu concentrations in the whole body of various species appear to be relatively uniform, averaging 2 mg/kg on a fat-free basis.

Evidence for an essential physiological role for Cu in animals was first provided by Hart et al. (1928) when they demonstrated that both Cu and Fe were required for hemoglobin formation in anemic rats. Three years later Cu

deficiency in grazing cattle was demonstrated in Florida (Neal et al., 1931) and by 1939 "neonatal ataxia" (swayback) of lambs (Bennetts and Chapman, 1937) and "falling disease" of cattle (Bennetts and Hall, 1939) were both recognized as Cu deficiency syndromes that could be prevented by Cu supplementation. Since then Cu has been shown to be essential for normal growth, iron metabolism, skeletal development, cardiovascular integrity, reproduction, pigmentation and keratinization, and development and function of the central nervous system.

The chance of a Cu deficiency occurring with resultant economic losses has resulted in most livestock diets being routinely supplemented with Cu. Because the effectiveness of such supplements depends on the utilization of the elements by the animal, careful consideration of the biological availability of supplements is required. However, attempts to determine Cu bioavailability from inorganic sources, using traditional methods, have met with limited success (Miller, 1983). Bioassays are complicated by Cu absorption and retention values that are low relative to that of other elements, by interactions between Cu and other dietary constituents, and by the fact that Cu does not have a radioactive isotope with a sufficiently long half-life to permit many of the metabolic studies required.

Recently, Black et al. (1984a, b) working with manganese (Mn) demonstrated that tissue uptake of Mn from high level, short term supplementation of dietary Mn was a

useful measure of relative bioavailability of inorganic sources. Reports of Cu accumulation in the tissues of animals fed high dietary levels of Cu (Dick, 1954; Hill and Williams, 1965; Norvell et al., 1975; Jensen and Maurice, 1979) suggest that this technique may be useful in the study of Cu bioavailability.

The experiments reported herein were conducted to determine if tissue Cu accumulation in sheep and chicks fed high dietary levels of Cu could be used as bioassay criterion for determining relative Cu bioavailability from inorganic sources.

CHAPTER II
REVIEW OF LITERATURE

Copper is a member of group IB of the periodic table of elements. These elements, differing essentially only in the number of electrons in the 3d shell, are known as transition elements. In addition to the metallic form, Cu can exist in the +1, +2, or +3 valence states, but more commonly occupies the +2 state (Miller, 1979). In natural mineral states, Cu occurs primarily as copper sulfide deposits in igneous rocks. In biological systems, the cuprous (Cu+) and cupric (Cu+2) forms predominate (Miller et al., 1979). The relative ease with which one form is converted to the other by addition or release of an electron gives Cu great versatility as an electron acceptor or donor (Frieden, 1968).

The presence of Cu in living matter was first demonstrated in the early 1800s when it was shown to be present in both plant and animal tissues (Miller et al., 1979). However, it was not until a century later that Cu was shown to be essential for animals (Hart et al., 1928). Copper has been shown to be an essential component of numerous oxidative enzyme systems including ascorbic acid oxidase, tyrosinase, uricase, cytochrome oxidase, monoamine oxidase and lysyl oxidase (Underwood, 1977). Copper concentrations in the whole body of various animal species average 2 mg/kg

on a fat-free basis, with newborn and young animals having higher concentrations than adults of the same species. The distribution of total body Cu among tissues varies with species, age and Cu status of the animal (Underwood, 1977).

Copper Metabolism

Absorption

Copper is absorbed from the stomach and small intestine of most animals; however, the site of maximal absorption varies slightly among species. In rats, Cu is absorbed in the stomach (Van Campen and Mitchell, 1965) and small intestine (Tompsett, 1940; Owen, 1964; Van Campen and Mitchell, 1965), with absorption being greatest in the stomach (Van Campen and Mitchell, 1965). The small intestine is the primary site of absorption in humans (Dunlap et al., 1974), pigs (Bowland et al., 1961), hampsters (Crampton et al., 1965) and chicks (Starcher, 1969). Considerable net absorption of Cu also occurs in the large intestine of sheep (Underwood, 1977).

The mechanism of Cu absorption is not precisely known. Present evidence indicates that intestinal absorption occurs in two steps. One step involves the mucosal uptake of Cu from the lumen, probably as a result of binding to sites on cell surfaces or within the cells. The second step involves transport to the serosal side and appears to be energy dependent (Crampton et al., 1965). These two steps appear

to be independent since anoxia and 2,4-dinitrophenol inhibit transport of Cu into serosal fluid, but have no effect on mucosal uptake.

The extent of Cu absorption is influenced by the amount and form of Cu ingested, by the dietary level of several other metal ions and organic substances, and by the age of the animal (Underwood, 1977). In a recent review, Cousins (1985) examined effects of various dietary constituents on Cu absorption and found that absorption is increased by amino acids, nitriloacetate, citrate, phosphate, gluconate, oxalate and ethylenediaminetetraacetic acid, while phytate, ascorbic acid, thiomolybdate, fiber, bile and zinc decrease absorption. The influence of dietary protein level on Cu absorption is not as clearly defined. In some studies, high protein diets have been shown to enhance Cu absorption (Engel et al., 1967; Greger and Snedeker, 1980), while in others, it appears to decrease absorption (McCall and Davis, 1961; Combs et al., 1966). Differences in protein digestibility may account for some of these differences, since digestibility could have a marked effect on the extent to which copper-amino acid and peptide chelates are formed (Cousins, 1985). Absorption of dietary Cu has been estimated to be between 40 and 60% in humans (Mason, 1979). In contrast, Cu absorption is estimated to be less than 10% in mature sheep (Suttle, 1972).

Transport

Plasma Cu is divided between two pools, one tightly and one loosely bound. The plasma cuproprotein ceruloplasmin represents the tightly bound pool that was thought to account for at least 90% of total plasma Cu in most species (Gubler, 1953). However, a recent report indicates that ceruloplasmin may account for only 60 to 65% of plasma Cu in rats and humans (Linder et al., 1985). The loosely bound pool is made up of albumin and amino acid complexes of Cu which are in equilibrium with each other and with ionic Cu (Neumann and Sass-Kortsak, 1967). There appears to be very little exchange between these two pools (Sternlieb et al., 1961). Once absorbed, Cu is complexed with albumin and amino acids and is transported via the hepatic portal vein primarily to the liver (Harris and Sass-Kortsak, 1967; Neumann and Sass-Kortsak, 1967). Recently, Linder et al. (1985) reported an additional Cu transport protein in rat and human serum. The protein, tentatively named "Transcuprein," has a greater affinity than albumin for Cu. However, in both in vitro and in vivo systems, it readily exchanges Cu with albumin. In addition, it was shown to donate its Cu to cultured cells more effectively than albumin. The presence of this protein in serum has yet to be confirmed by other researchers. Evans (1973) suggested that, although amino acid bound Cu represents only a small fraction of total plasma Cu, it may be a vital component of

the Cu transport mechanism since, in addition to facilitating Cu transport in blood, it also facilitates transport of Cu across a number of cell membranes. Upon arrival at the liver, a portion of complexed Cu is allowed to pass directly to systemic circulation, where it constitutes about 7% of plasma Cu. The remainder is taken up by hepatocytes and bound to metallothionein in the cytosol (Mason, 1979; Weiner and Cousins, 1980).

In liver, Cu can be stored, excreted in bile, or incorporated into newly synthesized ceruloplasmin which is discharged into plasma (Marceau and Aspin, 1972). The role of ceruloplasmin in Cu transport has only recently been demonstrated. In 1965, Owen demonstrated that Cu uptake by tissues is closely related to release of ceruloplasmin from liver, suggesting that ceruloplasmin transfers its Cu to those tissues. Further evidence for a transport role was furnished by Marceau and Aspin (1972) who showed that in rats, ceruloplasmin and albumin transfer their Cu to tissues of the body. Subsequently, Hsieh and Frieden (1975) found that ceruloplasmin Cu more quickly restored tissue cytochrome oxidase activity in deficient rats than did an equal amount of a Cu-albumin solution. More recently, Harris and DiSilvestro (1981) showed that activity of lysyl oxidase in chick aorta is directly correlated with ceruloplasmin content of plasma. Campbell et al. (1981) found that, in the rat, ceruloplamsin is the preferred plasma source of Cu for normal and malignant cells, and that

ceruloplasmin Cu turns over more rapidly than the protein moiety, a finding consistent with its role as a transport protein.

In summary, it appears that absorbed Cu is transported via the hepatic portal vein to liver complexed with albumin, amino acids and perhaps transcuprein. In liver, ceruloplasmin is synthesized and released into peripheral plasma. Plasma ceruloplasmin, albumin, amino acids and perhaps transcuprein then transport Cu to tissues of the body.

Excretion

In view of the relatively poor absorption of dietary Cu, it is not surprising that a high proportion of ingested Cu appears in feces. Although much of the fecal Cu represents unabsorbed Cu, excretion of Cu occurs chiefly via the biliary system (Gitlin et al., 1960; Beck, 1961; Bowland et al., 1961; Cartwright and Wintrobe, 1964a, b). In addition to being directly involved in Cu excretion, bile also appears to play an indirect role. Gollan et al. (1971) demonstrated the ability of bile to bind Cu and suggested that bile may also enhance Cu excretion by decreasing absorption. The ability to inhibit Cu absorption was demonstrated subsequently in rats (Gollan, 1975).

Under normal conditions, urinary excretion of Cu is minimal. In humans it is estimated to be approximately .5 to 3% of daily Cu intake (Cartwright and Wintrobe, 1964b). Evans (1973) attributed this to the fact that in blood the

majority of Cu is bound to ceruloplasmin or confined within erythrocytes and cannot permeate the glomerular capillaries. Urinary Cu excretion is increased in a number of human diseases, including renal diseases, proteinuria, cystinuria, diabetes and Wilson's disease (Owen, 1982b). In addition to fecal and urinary loss, negligible amounts of Cu are lost via sweat and normal menstrual flow.

Copper Deficiency and Functions

The manifestation of Cu deficiency varies with age, sex and species of animal, and with severity and duration of the deficiency (Underwood, 1977). Anemia is a common expression of Cu deficiency in all species when deficiency is severe or prolonged. Other Cu deficiency signs include skeletal and central nervous sytem abnormalities, depigmentation of hair and wool, infertility, cardiovascular diseases, diarrhea and immunodeficiency (Miller et al., 1979). In ruminants, physiological Cu deficiencies can be produced by four feeding situations: (1) high molybdenum (Mo), generally above 100 mg/kg diet, (2) low Cu:Mo ratio, 2:1 or less, (3) Cu deficiency, below 5 mg/kg diet and (4) high protein, 20 to 30% in fresh forage (Ward, 1978). This makes diagnosis and treatment of a Cu deficiency in ruminants difficult.

The physiological function of a micronutrient, such as Cu, can frequently be elucidated by studying the pathology that results from a deficiency of the nutrient (O'Dell, 1976). Most Cu deficiency diseases listed

previously can be related directly to a deficiency of specific Cu-dependent enzyme systems. These systems and their related diseases are discussed in the following sections.

Anemia and Iron Metabolism

The first evidence that Cu plays an essential role in animal physiology was provided by Hart et al. (1928) who showed that it is required for prevention of anemia. Since then, anemia has been found to be a common expression of Cu deficiency in all species. In rats, rabbits, pigs and lambs this anemia is hypochromic and microcytic, while in ewes and cattle it is hypochromic and macrocytic (Underwood, 1977). Anemias that are hypochromic and microcytic result from defects in hemoglobin synthesis. The causes of defective hemoglobin synthesis can be subdivided into three groups: (1) those affecting iron (Fe) metabolism, (2) those affecting biosynthesis of porphyrins and heme and (3) those affecting biosynthesis of globin (Lee et al., 1976). A role for Cu in the biosynthesis of globin has not been established to date.

Morphologic and biochemical similarities between Cu deficiency and anemia due to Fe deficiency led Lahey et al. (1952) to suggest that in Cu-deficient swine there is an abnormality in Fe metabolism. Evidence of abnormal Fe metabolism was demonstrated subsequently by Gubler et al. (1952). These researchers found that Cu-deficient pigs have

an impaired ability to absorb Fe, mobilize it from tissues and utilize it in hemoglobin synthesis. Further evidence for an impairment of mobilization from tissues was provided by Lee et al. (1968b) when they demonstrated failure of the duodenal mucosa, the reticuloendothelial system and hepatic parenchymal cells to release Fe to plasma at the same rate as control animals.

An important step toward an understanding of the role of Cu in Fe metabolism was made by Osaki et al. (1966) when they proposed that ceruloplasmin, by virtue of its ferroxidase activity, could enhance the rate of Fe saturation of transferrin and thus allow delivery of adequate amounts of Fe to erythropoietic sites. This proposal appeared to be validated when impairment in Fe metabolism was corrected by intravenous administration of ceruloplasmin to Cu-deficient pigs (Lee et al., 1968b; Roeser et al., 1970). Administration of ceruloplasmin to Cu-deficient pigs with normal Fe stores caused an increase in plasma Fe which was proportional to the log of ceruloplasmin dose. However, recent studies demonstrate, at least in rats, that it is possible to have a reduction in ceruloplasmin levels and still not develop anemia (Cohen et al., 1983; Fields et al., 1984).

In addition to its role in mobilization of Fe for hemoglobin formation, Cu appears to be essential for normal normoblast function. Lee et al. (1968a) found that Fe could not be incorporated into hemoglobin and, instead,

accumulated as nonhemoglobin Fe. Investigation of the heme biosynthetic pathway shows no reduction in activity of any heme biosynthetic enzymes. In fact, as anemia developed, activity of heme biosynthetic enzymes increased, appearing to indicate that Cu could not be a cofactor in these enzymes (Lee et al., 1968a). A possible explanation of the normoblast defect was proposed by Goodman and Dallman (1969) when they suggested that a Cu protein is required for normal mitochondrial Fe uptake, a step essential for heme synthesis. This proposal led a number of researchers to investigate the role of mitochondria in heme synthesis. Their observations indicate that an active, intact mitochondrial electron transport system is necessary for optimal synthesis of heme (Jones and Jones, 1969; Lemberg, 1969; Williams et al., 1976). Using Cu-deficient mitochondria with depleted cytochrome oxidase activity, Lee et al. (1976) demonstrated that these mitochondria consumed less oxygen and synthesized less heme than did normal mitochondria. Further, the rate of heme synthesis correlated with cytochrome oxidase activity. They therefore proposed that the defect in heme synthesis in Cu-deficient mitochondria is due to a deficiency of cytochrome oxidase. This proposal received support recently when Davies et al. (1985) demonstrated a marked decline in liver mitochondrial cytochrome oxidase activity in Cu-deficient rats.

Pigmentation and Keratinization

The Cu enzyme tyrosinase catalyzes oxidation of the amino acid tyrosine and other phenolic compounds to produce melanin, a pigment responsible for darkening of skin, hair and wool in most animals (Frieden, 1968). Mammalian tyrosinase contains between .22 to .25% Cu and has a molecular weight of about 33,000 daltons. Changes in hair and wool color are among the most characteristic manifestations of a Cu deficiency in sheep and cattle. In fact, lack of pigmentation in sheep has been reported to be a more sensitive index of Cu deficiency than anemia (Underwood, 1977). Decreased feather pigmentation has also been observed in chicks and poults fed Cu-deficient diets (Hill and Matrone, 1961; Savage et al., 1966). Tyrosinase has also been shown to be involved in albinism and a type of skin cancer called melanoma. In albinism, there is an absence or inactivity of tyrosinase, while in melanoma there is hyperactivity of tyrosinase (Frieden, 1968).

Impaired keratinization, a specific effect of Cu deficiency, is observed in a number of species. In sheep, it is characterized by failure of wool fibers to crimp. This failure is attributed to inadequate cross-linkage among fibers and a reduced number of disulfide bonds (Underwood, 1977). Copper supplementation of deficient sheep results in complete restoration of crimping.

Skeletal Abnormalities

Skeletal abnormalities resulting from Cu deficiency have been reported in most species. The abnormalities vary depending on species and on whether the deficiency is primary or secondary in nature. In dogs (Baxter and Van Wyk, 1953) and pigs (Follis et al., 1955), histological changes include thinned cortices, broadened epiphyseal cartilage and reduced osteoblastic activity. In chicks (Carlton and Henderson, 1964), the widened epiphyseal cartilages and thinner cortical bones observed in pigs and dogs are present, but the widened calcified cartilage matrix of the upper metaphysis is absent. Suttle et al. (1972) found that lambs born to, and suckled by, Cu-deficient ewes had none of the gross abnormalities observed in the dog, pig or chick. There was no evidence of widened epiphyseal cartilage observed in other species. The lesions observed were attributed to reduced osteoblastic activity. Bone abnormalities in cattle appear to differ depending on the type of Cu deficiency. In a primary deficiency, there appears to be a general matrix osteoporosis with decreased osteoblastic activity (Suttle and Angus, 1978), while in a secondary deficiency there is an irregular widening of epiphyseal cartilage in the presence of active osteoblasts (Irwin et al., 1974).

The defect in Cu-deficient bone is attributed to a failure of collagen cross-linking resulting from a decreased

activity of amine oxidases. Rucker et al. (1969) found that amine oxidase activity was reduced 30 to 40% in Cu-deficient chicks. In addition, bone collagen contained less aldehyde and was more soluble. Solubility of collagen was previously shown to be inversely related to the degree of cross-linking (Harding, 1965). Decreased cross-linking and aldehyde content of Cu-deficient collagen has also been observed by Chou et al. (1969). These researchers proposed that the catalytic role of Cu is mediated through an amine oxidase which catalyzes formation of an intermediate aldehyde compound which is involved in intramolecular cross-links. Further evidence for a role of Cu in cross-linking was provided by Rucker et al. (1975) when they demonstrated that Cu-deficient bones, which fractured with less deformity and torque than control bones, could be strengthened to that of control bones by addition of artificial cross-links.

Discovery of the amine oxidase, lysyl oxidase and the elucidation of its functions provided a greater understanding of the role of Cu in maintaining normal collagen cross-links. This Cu enzyme was shown to oxidatively deaminate lysyl and hydroxylysyl radicals in collagen, forming allysine and hydroxylysine (Narayanan, 1978; Rucker and Murray, 1978; Siegel, 1979). Allysine and hydroxylysine then condense with other peptide allysines, lysine or hydroxylysine residues to form cross-links (Opsahl et al., 1982). Failure to form adequate cross-links because of

reduced lysyl oxidase activity results in the skeletal abnormalities observed in most species.

Cardiovascular System

Cardiovascular defects associated with Cu deficiency have been reported in cattle (Bennetts et al., 1948; Leigh, 1975), swine (Gubler et al., 1957; Shields et al., 1962), chicks (O'Dell et al., 1961; Carlton and Henderson, 1963), turkeys (Savage et al., 1966; Simpson et al., 1971), rabbits (Hunt and Carlton, 1965), rats (Abraham and Evans, 1972; Kelly et al., 1974; Wallwork et al., 1985) and guinea pigs (Everson et al., 1967). Cardiac hypertrophy, a common pathology of Cu deficiency, has been observed in cattle (Leigh, 1975), swine (Gubler et al., 1957; Shields et al., 1962) and rats (Abraham and Evans, 1972; Kelly et al., 1974; Wallwork et al., 1985). Associated with cardiac hypertrophy is an alteration in cardiac mitochondrial morphology accompanied by decreased cytochrome oxidase activity. Cytochrome oxidase is the terminal link in the electron transport chain (Green, 1956) and is found primarily in mitochondrial membranes of most tissues (Gallagher and Reeve, 1976; Chance and Leigh, 1977). Reduced tissue cytochrome oxidase appears to be a consistent finding in Cu-deficiency (Owen, 1982a). In cattle, a condition known as falling disease is characterized by sudden deaths, usually after mild exercise or excitement. The disease believed due to acute heart failure results from

degeneration of the myocardium with replacement fibrosis (Underwood, 1977).

Extensive hemorrhaging resulting from rupture of major blood vessels is observed in chicks (O'Dell et al., 1961), pigs (Shields et al., 1962) and guinea pigs (Everson et al., 1967). In all cases cardiovascular tissues appear to lose the ability to maintain normal elasticity. Starcher et al. (1964) found that aortas of Cu-deficient chicks contained only 50% as much elastin as did control chicks by day 17. O'Dell et al. (1966) found that Cu-deficient aortas contained less elastin and more soluble collagen than did controls. Copper-deficient elastin contained 40% less desmosine and isodesmosine and five times as much lysine as did the control protein. In Cu-deficient pigs (Shields et al., 1962), a derangement of elastic membranes results in a 60% decrease in the tensile strength of thoracic aortas. In rabbits (Hunt and Carlton, 1965), elastin defects consist of loss of elastic fibers and calcification and fragmentation of internal elastic membranes in muscular and elastic arteries.

Observations of reduced elastin synthesis and changes in the nature of the deficient protein suggested a role for Cu in elastin synthesis. Elucidation of such a role began with the observation that Cu-deficient elastin contained a high concentration of lysine, but a low concentration of desmosine and isodesmosine (Starcher et al., 1964; Miller et al., 1965; O'Dell et al., 1966). Desmosine and its

isomer isodesmosine are tetracarboxylic tetra-amino acids involved in elastin cross-linkage (Thomas et al., 1963). Based on structure, Partridge et al. (1964) suggested that both amino acids could arise from condensation of four lysine residues pre-existing in straight chain elastin precursors. In order for condensation to occur the epsilon amino group of lysine would have to be oxidatively deaminated to an aldehyde. Such a reaction is catalyzed by amine oxidases. Kim and Hill (1966) examined this hypothesis and found that Cu deficiency resulted in reduced oxidase activity in aortas. Addition of amine oxidase to Cu-deficient systems decreased lysine-to-desmosine ratios to approximately that of Cu supplemented systems. The cuproenzyme, lysyl oxidase, was subsequently shown to be the amine oxidase that catalyzes the oxidative deamination of lysine to give allysine, the aldehyde derivative. In elastin, three allysines combine with one lysyl to form desmosine (Owen, 1982b).

In summary, the role of Cu in cardiovascular integrity involves two Cu-dependent enzymes, cytochrome oxidase and lysyl oxidase. Cytochrome oxidase is required for cardiac mitochondrial integrity and lysyl oxidase is required for synthesis of normal elastin.

Central Nervous System

Nervous disorders resulting from Cu deficiency are observed in neonates of several species including sheep

(Bennetts and Chapman, 1937; Innes and Shearer, 1943; Barlow et al., 1960a, b), goats (Owen et al., 1965; O'Sullivan, 1977; Inglis et al., 1986), rats (Carlton and Kelly, 1969; Dipaolo et al., 1974), pigs (Joyce, 1955; Wilkie, 1959; McGavin et al., 1962) and guinea pigs (Everson et al., 1967, 1968). The disorder observed most frequently occurs in lambs and goats and has been termed neonatal ataxia or "swayback." Swayback is characterized by motor dysfunction of the hind limbs and is associated with subnormal levels of Cu in tissues of ewes and lambs (Underwood, 1981). Two forms of swayback occur in lambs and goats, a congenital form in which the animals are affected at birth and a delayed form in which signs may not appear for several weeks.

The pathology of swayback was initially characterized as a diffuse symmetrical demyelination of the cerebrum, with liquefaction and cavitation being a common end stage of the lesion (Innes and Shearer, 1943). This characterization of swayback was challenged by Behrens and Schulz (1959) and Schulz and Behrens (1960) who suggested that the disorder resulted from venous stasis, edema, perivascular softening and necrosis. Barlow (1958) and Barlow et al. (1960b) found that although lesions of cerebral white matter were almost always pathogonomic for swayback, they were not an essential part of the disease. They concluded that neuronal hyaline necrosis and fiber degeneration in the brain stem and spinal cord was the essential pathology of swayback.

Recently, Howell et al. (1981) investigated cases of congenital and delayed swayback and proposed a possible explanation for observed differences in the pathology of the disease. They proposed that pathological lesions would differ depending on when the Cu deficiency occurred. A Cu deficiency in early or mid pregnancy results in cerebral lesions, since maximum cerebral growth is occurring during this period. In late pregnancy, Cu deficiency results in lesions of the cerebellum and of myelinated structures in the cerebrum and cord. Copper deficiency occurring after parturition affects the spinal cord most severely, since the spinal cord of the lamb more than doubles in size from 5 weeks of age to maturity. There appears to be some support for such a proposal since swayback can be prevented either by supplementing pregnant ewes (Allcroft et al., 1959) or by supplementing deficient lambs of highly susceptible populations (Lewis et al., 1981).

Innes and Shearer (1943), after discussing the possibility of myelin aplasia being involved in the pathogenesis of swayback, concluded that the disease was the result of demyelination. This conclusion appeared to receive support from Barlow (1963) who showed that myelin present in the brain of newborn lambs disappeared with age while products indicative of myelin degeneration increased. However, in the same study, Barlow (1963) pointed out that failure of the mechanism of myelin fabrication or myelin degeneration might both equally well account for the pathological

lesions seen. Since then several researchers have presented evidence in favor of myelin aplasia. Howell et al. (1964) found less myelin in the central nervous system of swayback lambs and, since no products of myelin degeneration were detected, concluded that the lesion was the result of myelin aplasia. Everson et al. (1968), using the guinea pig as a model, concluded that Cu deficiency affects normal development of myelin throughout the brain. There was no evidence of myelin degeneration. Dipaolo et al. (1974) found histological evidence for decreased myelination and demonstrated significant reductions in typical myelin lipids in Cu-deficient rats. Zimmerman et al. (1976) found a 56% decrease in yield of myelin per gram of brain tissue in 22-day-old rats whose dams were Cu-deficient. The absence of cholestrol ester in myelin from Cu-deficient animals suggested that there was no myelin degeneration.

Observations of abnormal myelination in Cu-deficient animals implied a role for Cu in myelin synthesis. Gallagher et al. (1956a, b) provided the first evidence for such a role when they demonstrated loss of activity of the cuproenzyme cytochrome oxidase and suppression of phospholipid synthesis in Cu-deficient rats. As a direct result of these observations, Howell and Davidson (1959) examined brain tissue from swayback lambs and found subnormal levels of Cu and cytochrome oxidase. Barlow (1963) confirmed these findings and also found that enzyme activity was lowest in those sites in which lesions of the disease appear.

Similar findings led Fell et al. (1965) to suggest that a low Cu content in brain leads to a deficiency of cytochrome oxidase. Gallagher and Reeve (1971) supplied the final evidence when they postulated that loss of cytochrome oxidase activity leads to depressed phospholipid synthesis by liver mitochondria, by interfering with the provision of endogenous ATP to maintain an optimal rate of synthesis. This hypothesis received support recently when Davies et al. (1985) found that ATP and ADP contents of Cu-deficient mitochondria were reduced by 39 and 40%, respectively. Depressed phospholipid synthesis results in decreased myelin synthesis since myelin is composed largely of phospholipids.

In addition to its role in myelination, Cu is required for biosynthesis of the catecholamine neurotransmitters, dopamine and norepinephrine. Significant depression in catecholamine concentrations has been observed in ataxic lambs (Hunt and Johnson, 1972). Parkinson's disease, an extapyramidal disorder involving motor dynfunction in humans is associated with depressed dopamine levels (Hornykiewicz, 1973). These observations suggest that impaired catecholamine synthesis may be involved in the locomotor disorder of swayback.

The precise mechanism by which Cu deficiency depresses catecholamine synthesis is unknown. Tyrosine hydroxylase, the rate limiting enzyme in dopamine synthesis, is significantly depressed in Cu deficiency (Morgan and O'Dell, 1977). The enzyme does not appear to be Cu-dependent (Nagatsu

et al., 1964) and its activity is not restored by Cu repletion (Feller and O'Dell, 1980). This led Feller and O'Dell (1980) to suggest that impaired dopamine metabolism is due to a neuron development defect and not to a catalytic dysfunction.

Norepinephrine is synthesized from dopamine in a reaction catalyzed by the cuproenzyme dopamine-b-hydroxylase (DBH). Depressed DBH activity could account for the low nonepinephrine levels observed in Cu deficiency. However, current evidence appears to indicate that this is not the case. Dopamine-b-hydroxylase activity was found to be significantly elevated in brains of rats made Cu-deficient either by dietary means or as a result of a genetic defect (Hunt, 1977; Prohaska and Smith, 1982). Hesketh (1980, 1981a, b) investigated the effect of Cu deficiency on adrenal glands of cattle and rats. In both species there is a reduction in norepinephrine levels. Dopamine-b-hydroxylase activity, however, is elevated in rats but is unchanged in cattle. Hesketh (1981b) concluded that depressed norepinephrine levels cannot be accounted for only by reduced DBH activity but that it may be due to defective storage, increased catabolism or reduced synthesis of the catecholamine. Prohaska and Smith (1982) provided a possible explanation for increased DBH activity. They suggested that Cu deficiency, both dietary and genetic, leads to reduced DBH activity in vivo, resulting in reduced norepinephrine synthesis. However, depletion of

norepinephrine induces more apo-DBH synthesis, which is then converted to DBH in vitro by trace amounts of Cu used in the DBH assay. This explanation appears plausible since Hesketh et al. (1985), using radioisotopes to study in vivo catecholamine synthesis, concluded that DBH activity is reduced in the norepinephrine-synthesizing cells of the adrenal medulla of Cu-deficient rats. At present, there is insufficient evidence to determine whether lower catecholamine levels are causally related to swayback.

Reproduction

Impaired reproduction resulting from Cu deficiency was first reported in rats by Keil and Nelson (1931). Rats fed a milk diet, or a milk diet plus Fe produced only one litter in 5 months. In comparison, rats fed the same milk and Fe diet supplemented with .5 mg of copper sulfate daily produced an average of five litters. Dutt and Mills (1960) also reported reproductive failure in Cu-deficient rats. Out of 18 rats fed a low Cu diet, only one produced live young. The rats cycled and conceived normally but then resorbed their fetuses. In addition to fetal resorption, Hall and Howell (1969) reported depigmentation of hair and incisor teeth and a 90% reduction in liver Cu concentrations of deficient rats.

Investigation of fetal resorption indicates that fetuses and placentas of Cu-deficient rats develop normally until day 13 of pregnancy. On day 13, fetal necrosis occurs

and is followed by placental necrosis on day 15. No lesions were observed in the maternal ovaries, mammary or pituitary glands (Howell and Hall, 1969). Fetal resorption appears to occur only in severe cases of Cu deficiency. O'Dell et al. (1961) were able to obtain litters from rats reared on a low Cu diet containing about 1 mg/kg Cu, but the offspring were severely anemic and nonviable. Lesions observed in the newborn included severe edema, wide-spread subcutaneous hemorrhage, skeletal abnormalities and abdominal hernias.

Copper deficiency in poultry results in egg abnormalities and reduced hatchability. Laying hens fed a Cu-deficient diet (.7-.9 mg/kg) for 20 weeks produced fewer eggs and had subnormal concentrations of Cu in plasma, liver and eggs. Hatchability of eggs dropped rapidly and by week 14 approached zero (O'Dell, 1968). Reduced hatchability was attributed to early embryonic mortality. Copper-deficient embryos were anemic and had a high incidence of hemorrhage after 72-96 hours of incubation. Embryonic mortality was also observed by Simpson et al. (1967). Feeding a Cu-deficient diet (2 mg/kg) to laying hens resulted in mortality of all embryos within 10 days. Microscopic examination revealed that cells in the tunica media of Cu-deficient embryos were immature and elastin synthesized was abnormal. Baumgartner et al. (1978) fed hens a diet containing .72 mg/kg Cu and observed that of eggs produced, 10% were shell-less and 40% were malformed. There was a significant reduction in shell weight and thickness and

amino acid analysis of shell membranes indicated increased lysine content.

Reproductive anomalies have also been reported in Cu deficient ruminants. One of the first reports to implicate Cu deficiency in reproductive disorders of ruminants was that of Bennetts and Chapman (1937). They reported that neonatal ataxia, a disease affecting lambs during gestation, was caused by Cu deficiency and could be prevented by Cu supplementation of pregnant ewes. Since then there have been a number of reports associating Cu deficiency with reproductive disorders. Howell (1968) reported that of nine ewes fed a synthetic Cu-deficient diet, five died without becoming pregnant, two aborted small, dead, immature fetuses and two failed to conceive by the end of the experiment. Anke (1973) found that Cu-deficient goats not only had lower conception rates, but of those conceiving, 50% aborted. Aborted fetuses were mummified and there was placental necrosis. Wiener and Sales (1976) reported that Cu deficiency did not appear to affect libido of rams, but rams having higher plasma Cu concentrations appeared to be more fertile.

The role of Cu in bovine reproduction is controversial. Early reports appeared to indicate a negative relationship between Cu deficiency and fertility (Allcroft and Parker, 1949; Blakemore and Venn, 1950; Becker et al., 1953; Munroe, 1957). Low fertility was associated with reduced conception rates resulting from delayed or depressed estrus.

Recent reports, however, implicate high molybdenum concentrations rather than Cu deficiency (Case et al., 1973; Peterson and Waldren, 1977; Phillippo et al., 1982, 1985). Phillippo et al. (1985) showed that a diet containing 5 ppm molybdenum can delay the onset of first estrus in cattle by 6-8 weeks. Associated with delayed estrus was a 50% decrease in conceptions. These observations suggest that earlier reports of impaired fertility in cattle may well be molybdenum-induced, since the studies were conducted in soils known to be high in molybdenum.

Studies relating blood Cu status to fertility are also inconclusive. Some researchers found no relationship between blood Cu and fertility (Rowlands et al., 1977; Larson et al., 1980; Phillippo et al., 1982; Whitaker, 1982) while others observed reduced fertility in animals exhibiting low blood Cu concentrations (Bennetts et al., 1948; Allcroft and Parker, 1949; Blakemore and Venn, 1950; Munroe, 1957). Similarly, Cu supplementation improved fertility in some herds (Blakemore and Venn, 1950; Munroe, 1957; Pickering, 1975; Hunter, 1977) but had no effect in others (Poole and Walshe, 1970; Phillippo et al., 1982; Whitaker, 1982). These results indicate the need for definitive studies on the role of Cu in bovine reproduction.

The precise lesions responsible for reproductive abnormalities are yet to be elucidated. However, observations of fetal death associated with hemorrhaging and placental necrosis suggest decreased vascular integrity of

blood vessels in the placenta and fetus. The role of Cu in maintaining cardiovascular integrity has been discussed previously.

Copper Toxicosis

Copper, although an essential element, can be toxic if consumed in excess. Attempts to determine maximum tolerable levels are complicated by numerous interrelationships among Cu and other trace elements and substances. The situation is further complicated by differences in susceptibility between ruminants and nonruminants. In general, ruminants are more susceptible to Cu toxicosis than nonruminants. Like Cu deficiency, the basis for Cu toxicosis may well represent diminished enzymatic activity since ionic Cu inhibits a number of enzymes (Owen, 1981). The present review will be limited to those species in which Cu toxicosis has been reported to occur under practical feeding conditions.

Reports of Cu toxicosis in swine first appeared in the late 1950s. Not coincidentally, these reports began to appear shortly after it was demonstrated that daily gains of growing pigs were significantly improved by feeding 250 mg/kg Cu as copper sulfate (Barber et al., 1955a, b; Bowler, 1955). Gordon and Luke (1957) diagnosed Cu toxicosis as cause of death in a pig submitted for postmortem examination. Necropsy revealed a generalized icterus and all organs and tissues were stained an intense yellow.

Dietary Cu concentrations were unknown but liver analysis revealed a Cu content of 2500 mg/kg on a dry matter basis (DM). O'Hara et al. (1960) fed pigs a diet containing 250 mg/kg Cu as copper sulfate and reported incidences of parakeratosis and a syndrome thought to be chronic Cu toxicosis. Lesions were typical of chronic Cu toxicosis reported in other species except for the absence of icterus and hemoglobineria. Liver Cu concentrations of affected pigs varied between 684 and 1800 mg/kg (DM). Copper levels of 250 mg/kg and above also proved toxic in weanling pigs fed a typical corn-soybean meal diet (Wallace et al., 1960). However, in pigs fed a barley, wheat and fish meal diet, Cu toxicosis did not occur until dietary levels exceeded 500 mg/kg Cu (Allcroft et al., 1961). Buntain (1961) found that dietary Cu levels as low as 130 mg/kg may cause toxicosis under certain conditions. During a 5 to 6 week period, 23 fattening pigs were lost from a herd of 200. Postmortem examination revealed evidence of icterus, liver necrosis and kidney damage. Liver Cu concentrations ranged from 1500 to 2200 mg/kg (DM).

Failure to establish an absolute toxic level for Cu in swine suggests that factors other than dietary Cu level may also influence the onset of toxicosis. Dietary levels of zinc (Zn) and Fe are two such factors. Suttle and Mills (1966a, b) reported that addition of 150 mg/kg Zn and 150 mg/kg Fe to diets containing 250 or 425 mg/kg Cu prevented Cu toxicosis that developed when Zn and Fe were not added.

In swine fed 750 mg/kg Cu addition of 750 mg/kg Fe completely eliminated signs of toxicosis, while 500 mg/kg Zn eliminated all signs except that of anemia. DeGoey et al. (1971) found that Zn and Fe at dietary levels of 100 mg/kg prevented signs of toxicosis in swine fed 500 mg/kg Cu. The interaction of Cu, Zn and Fe in mammalian species has been reviewed extensively by Davis (1980). He concluded that diets that are either deficient in or contain excessive levels of Cu, Zn or Fe will cause changes in the absorption of the elements from the gastrointestinal tract. In swine, Zn and Fe appear to alleviate Cu toxicosis by causing a reduction in Cu absorption.

Suttle and Mills (1966a) investigated the etiology of Cu toxicosis in swine. Liver damage was a consistent finding in affected animals. The damage to liver tissue did not appear to be caused directly by excessive storage of Cu, since Cu concentrations in livers of affected animals were similar to that of healthy Cu-supplemented animals. They proposed that liver damage occurs when serum Cu levels exceed 300 ug/100 ml. Below this level, liver is capable of removing Cu from blood and storing it. Above this level, liver tissue is probably damaged by excess Cu arriving at the liver. This critical level was chosen since it was found to be exceeded frequently in pigs with Cu toxicosis, but rarely exceeded in healthy animals.

Copper toxicosis in poultry was investigated by several groups. Pullar (1940) found the minimum lethal dose (MLD)

of Cu varied from 300-1500 on a mg/kg live body weight basis, depending on class of poultry and form of Cu fed. The maximum daily intake of copper carbonate tolerated by birds was determined to be 60 mg/kg live body weight for chicks, and 29 mg/kg live body weight for ducks. Overall, ducks proved to be more sensitive to Cu toxicosis than were pigeons or chickens. Goldberg et al. (1956) fed adult leghorn hens increasing amounts of copper acetate (28-56 mg/kg live body weight) and observed weight loss, hemolytic anemia and mortality within 5 weeks. Laying hens fed diets containing more than 300 mg/kg Cu produced fewer eggs and had lower liver lipid concentrations compared to controls (Jackson and Stevenson, 1981a, b). Mayo et al. (1956) reported reduced performance and muscular dystrophy in chicks fed a corn-soybean meal diet supplemented with 324 mg/kg Cu. Smith (1969) observed a 7.5% reduction in live weight gain of chicks fed 350 mg/kg Cu. In contrast, Mehring et al. (1960) found 500 mg/kg Cu to be the minimum toxic level in chicks fed a corn-soybean meal diet, while Arthur et al. (1958) found no signs of toxicosis in chicks fed 500 mg/kg Cu. Turkey poults appear to be more tolerant of Cu than chicks. Wiederanders (1968) injected three groups of poults with .5 mg Cu/day for up to 84 days and was unable to produce Cu toxicosis until the dose was increased to 5 mg/day for a further 17 days. Vohra and Kratzer (1968) reported no deleterious effects in turkey poults fed 676 mg/kg Cu for 21 days. Christmas and Harms (1979) observed

reduced performance in poults fed 500 mg/kg Cu for 21 days, but attributed this to the fact that the basal diet was deficient in sulfur amino acids.

Like swine, dietary factors other than absolute Cu levels may dictate the onset of Cu toxicosis in poultry. Sulfur amino acid content of the diet appears to be of prime importance. Several researchers (Jensen and Maurice, 1979; Christmas and Harms, 1979, 1984; Ekperigin and Vohra, 1981a) have demonstrated the ability of methionine to alleviate the growth depressing effect of Cu. Robbins and Baker (1980a, b) reported that feeding chicks 250 and 500 mg/kg Cu increased their sulfur amino acid requirement by 13 and 30%, respectively. Thus, minimum toxic levels of Cu may have to be defined in terms of sulfur amino acid content of diets.

Of all species, sheep are the most susceptible to Cu toxicosis and can be affected at any age although most reported cases occur in mature animals (Todd, 1969). Young calves appear to be as susceptible as sheep, but become more tolerant as they mature. With the exception of experimentally induced Cu toxicosis (Kidder, 1949; Shand and Lewis, 1957; Weiss and Baur, 1968) and a few cases of acute toxicosis (Garner, 1961), there are very few reports of chronic Cu toxicosis occurring in cattle under natural grazing conditions. This led Todd (1969) to conclude that from a nutritional point of view, chronic Cu toxicosis can be considered as being almost entirely confined to sheep.

Causes of Cu toxicosis in sheep are myriad and include oversupplementation of Cu, accidental use of high-copper swine feed, use of Cu-based fungicides, overtreatment of Cu deficiency and grazing of pastures deficient in molybdenum or high in heliotropic species (Owen, 1981).

Copper toxicosis can be acute or chronic. Signs of acute toxicosis include nausea, salivation, violent abdominal pain, convulsions, paralysis, collapse and death. Lesions observed on postmortem examination include gastro-enteritis, necrotic hepatitis, splenic and renal congestion and evidence of antemortem intravascular coagulation (NRC, 1980). In cases of acute toxicosis, death can occur within 24 to 72 hours (Isael et al., 1969; Wiener and MacLeod, 1970) or may be delayed 3 to 7 days (Macleod and Watt, 1970; Sasu et al., 1970) depending on Cu dose and mode of supplementation.

In contrast, cases of chronic toxicosis may not result in mortality for several months. Chronic Cu toxicosis can be divided into three distinctive phases, pre-hemolytic, hemolytic and post-hemolytic. In the pre-hemolytic phase, which can last from weeks to several months, signs of the disease are absent, but Cu accumulates predominantly in liver, and isolated hepatic parenchymal cells die liberating their enzymes into the bloodstream (Howell and Kumaratilake, 1985). Hepatic Cu concentrations as high as 4000 mg/kg on a dry matter basis have been reported (Gooneratne et al., 1979). During the latter part of the phase there are marked

increases in serum concentrations of several enzymes including glutamic oxaloacetic transaminase, lactic dehydrogenase, glutamic dehydrogenase, sorbitol dehydrogenase and arginase (Owen, 1981). Serum concentrations of Cu remain normal until the hemolytic crisis is imminent, then sharply increase (Eden, 1940; Todd and Thompson, 1963; Ishmael et al., 1972). The transition from the pre-hemolytic to the hemolytic phase is abrupt. Animals undergoing a hemolytic crisis become dull, refuse to eat and are excessively thirsty. There is a marked increase in blood Cu which causes hemolysis, jaundice, methemoglobinemia, hemoglobinuria and extensive necrosis of liver and kidney tissue (Miller, 1979). In addition, there is evidence of brain (Doherty et al., 1969; Howell et al., 1974) and muscle (Thompson and Todd, 1974; Gooneratne and Howell, 1980) damage. During the post-hemolytic phase, if the animal survives, reparative changes commence but further episodes of hemolysis can occur even if the source of Cu is removed (Ishmael et al., 1971).

Recently, Gooneratne and Howell (1985) investigated the cellular pathology of hepatic and renal cells in chronic Cu poisoning of sheep and proposed the following pathogenesis

> During Cu loading most of the excess Cu is stored in liver lysosomes. In the early stages, liver accommodates for increased hepatic Cu by proliferation of its lysosomes. Beyond a 'critical' hepatic Cu concentration, the rate of proliferation of lysosomes decreases and excess Cu is found in enlarged, irregular lysosomes. At a further 'critical' Cu concentration the liver lysosomes can no longer handle the increased hepatic Cu

> uptake and they may rupture releasing Cu and acid hydrolases into the cytoplasm, with resultant liver cell death. Some of the Cu from the released liver lysosomes enters the bloodstream where it is transported in the plasma. Some of this Cu enters the cells of the proximal convoluted tubules. The entry of Cu and acid hydrolases into the already Cu loaded cells of the proximal convoluted tubules may overload their lysosomal system and be directly responsible for the death of these cells. (1985, p. 189)

Diagnosis of Cu toxicosis is a prerequisite for treatment of the condition. Ross (1966) demonstrated the value of the glutamic oxaloacetic transaminase assay as an aid in the diagnosis and assessment of treatment of Cu toxicosis in lambs. Once diagnosed, appropriate remedial steps can be taken. The interaction among copper, molybdenum and sulfate in ruminants has provided a basis for treatment of Cu toxicosis. Dick (1956) reported that dietary sulfate potentiated a copper-molybdenum antagonism which depressed tissue Cu concentrations in ruminants. The precise mechanism of interaction among these elements is still being investigated but the hypothesis proposed by Suttle (1974b) seems plausible. Dietary molybdate is thought to react with sulfide (provided by reduction of sulfate) in the rumen to form thiomolybdate. Thiomolybdate thus formed can then interact with Cu either in the rumen or in tissues to form complexes in which Cu is unavailable. Formation of such complexes should undoubtedly prove helpful in alleviating Cu toxicosis. Reports indicate that this may indeed be the case. Suttle (1979) fed thiomolybdate to lambs and found

that hepatic accumulation of Cu was decreased by about 30% in animals supplemented with Cu. Gooneratne et al. (1981a) found that intravenous injections of 100 mg of ammonium tetrathiomolybdate twice daily not only prevented hemolysis but also prevented further crises in sheep already in hemolysis. The authors concluded that chronic Cu toxicosis can be successfully prevented or treated by intravenous injections of appropriate doses of ammonium tetrathiomolybdate.

Copper Bioavailability

Modern analytical techniques and instrumentation make it possible for nutritionists to accurately determine trace element concentration in animal tissues and feeds. Unfortunately, however, such determinations provide no information on utilization of the elements by animals. Utilization involves the concept of bioavailability and is critical in the selection of a suitable source of the element. In terms of trace elements, bioavailability may be defined as the proportion of an ingested element that is absorbed, transported to its site of action, and converted to the physiologically active species (O'Dell, 1983). Traditionally, trace element bioavailability studies have been conducted at deficient dietary levels, usually with purified or semipurified diets. The mineral sources are then added at graded levels and response criteria measured. In general, the bioavailability of an element in a

particular source is determined relative to its functional availability from a standard source. Use of a standard source allows expression of bioavailability in terms of relative biological availability (Miller, 1983). Since cases of Cu toxicosis and deficiency are both often reported in the literature, it is evident that the relative bioavailability of Cu sources is of major concern.

Copper bioavailability studies were first conducted using anemic rats. In these studies hemoglobin regeneration was used as the response criterion. Schultze et al. (1934, 1936) found that Cu from copper caseinate, copper aspartate, copper citrate, copper nucleinate, copper pyrophosphate, cysteine cuprous mercaptide, wheat germ, brewers' yeast, porcine liver and heart was readily utilized while that from copper hematoporphyrin was not. In a comparison of Cu salts, Cu from copper sulfate, cuprous oxide, cuprous iodide and copper hydroxide was readily utilized by anemic rats. Copper from cupric sulfide was found to be less efficiently utilized (Keil and Nelson, 1931; Sherman et al., 1934). In addition to hemoglobin regeneration, melanin-pigment regeneration, growth and liver Cu storage in rats have also been used as indexes of bioavailability. Mills (1955, 1956) reported that Cu from aqueous extracts of herbage was more available than Cu from copper sulfate. The author concluded that the greater availability of Cu from herbage extracts resulted from more efficient transport of herbage extract Cu through the intestinal mucosa.

In studies with mature rats and using liver Cu storage as the criterion, Nesbit and Elmslie (1960) found copper oxide and copper pyrophosphate to be 27 and 49% as available, respectively, as was copper sulfate. Lo et al., (1984) evaluated availability of Cu from isolated soybean protein and found it to be as available as Cu from a standard copper carbonate source. Serum and liver Cu concentrations were both effective indexes of bioavailability. Price and Chesters (1985) recently reported the development of a new bioassay for assessment of Cu availability. The technique involves a period of depletion followed by repletion and is based on the observation that increases in cytochrome oxidase activity in the duodenal mucosa of partially Cu depleted rats is linearly related to Cu dose. Using this technique, the authors found that Cu from untreated dried grass, rumen, duodenal and ileal digesta of sheep were 75, 12, 43, and 28% as available, respectively, as that of copper sulfate.

Compared to rats, there are fewer reports of Cu bioavailability studies in poultry. Willingham and Hill (1970) compared oxide and carbonate forms of Cu to that of the sulfate form. They found that chicks fed the sulfate form grew faster and had higher liver Cu and hemoglobin concentrations at day 21, than those fed oxides or carbonates. McNaughton et al. (1974) found cuprous oxide and cuprous iodide to be 76 and 82% as available, respectively, as copper sulfate, in broilers fed test diets for 21 days.

Physiological indicators used to determine bioavailability included hematocrit, hemoglobin and liver Cu concentrations. Norvell et al. (1975) fed graded levels of Cu (120-720 mg/kg) as the acetate, chloride and sulfate salts to broiler chicks for periods up to and including 8 weeks of age. They found that Cu in the acetate, chloride and sulfate forms depressed gains and increased Cu residues in liver, muscle and kidney tissue. Comparable levels of Cu fed as the oxide salt had no effect on these parameters, indicating that Cu oxide was less available than the other salts.

As with poultry, few Cu bioavailability studies have been conducted in swine. Copper as cupric sulfate is more efficiently absorbed and stored in porcine liver than is Cu as cupric sulfide (Barber et al., 1961; Bowland et al., 1961; Cromwell et al., 1978). Buescher et al. (1961) fed pigs radioactive Cu in the form of cupric carbonate, cupric sulfate or cuprous oxide and based on serum uptake and excretion of radiocopper concluded that the biological availabilities of the three forms were similar for swine. Copper carbonate was found to be equally as effective as copper sulfate in promoting growth in pigs fed 250 mg/kg Cu, but was less effective in promoting liver Cu storage (Allen et al., 1961). In weanling pigs fed 250 mg/kg supplemental Cu as copper sulfate, copper oxide or copper carbonate, liver Cu concentrations were highest in those fed sulfate (218 mg/kg), lowest in those fed oxide (32 mg/kg), and

intermediate in those fed carbonate (89 mg/kg; Bunch et al., 1961, 1965).

Copper bioavailability studies in ruminants have been conducted in both sheep and cattle. Lassiter and Bell (1960) used sheep to determine the relative availability of ^{64}Cu from cupric sulfate, cupric chloride, cupric oxide needles and powder, cuprous oxide, cupric nitrate, cupric carbonate and copper wire. Copper from the powdered oxide was more available than from the needles, while Cu from wire was essentially unavailable. Cupric sulfate, cupric chloride and cupric nitrate had similar availabilities. Copper absorption was found to be higher from oral doses of cupric carbonate than from cupric or cuprous oxide. Suttle (1974a), noting that conventional balance techniques are inadequate to determine Cu availability because only a small proportion of Cu ingested by sheep is absorbed (10% or less) and differences in absorption can be masked by analytical errors (Mills and Williams, 1971), proposed the use of a depletion-repletion technique. With this technique depleted sheep are repleted with oral and intravenous doses of Cu. The relative responses of plasma Cu to oral and intravenous Cu is then used to estimate true availability of dietary Cu. Using this technique Suttle (1974) estimated the availability of dietary Cu to be 4.1% in one experiment, while in a second experiment availabilities ranged from 4.5 to 11.4%. The author concluded that this technique provides a sensitive method for assessing the availability of Cu in

ruminant diets. Chapman and Bell (1963) used a radioisotope technique to evaluate the relative absorption and excretion of Cu from various sources by beef cattle. Copper from cupric nitrate, cupric sulfate and cupric chloride was absorbed to a similar extent. Cupric carbonate had the highest rate of absorption but also had the highest rate of excretion, indicating that retention may be a problem. Copper from powdered cupric oxide was absorbed to a greater extent than Cu from cupric oxide needles. Absorption, although not a true measure of bioavailability, is a major component of bioavailability and as such does provide an indication of bioavailability. The most recent report of Cu bioavailability in ruminants was that of Kincaid et al. (1986) who reported that Cu from copper proteinate was more available than Cu from copper sulfate for calves fed diets containing copper-molybdenum ratios varing from .9 to 2.8.

In addition to in vivo studies, several in vitro techniques have been developed in an attempt to predict bioavailability. Solubility tests have proved by far to be the most effective. Solvents evaluated include distilled water, hydrochloric acid, citric acid and neutral ammonium citrate. Of these, the hydrochloric acid test has become one of the most widely used procedures in the feed industry (Bernhardt, 1986).

Information presented in this review demonstrates not only the essentiality of Cu, but also the consequences of deficiency and toxicity. It is also evident that although

relative bioavailability of Cu sources is of major concern, relatively few Cu bioavailability studies have been conducted. Mills and Williams (1971) may have provided an explanation for this when they noted that conventional balance techniques are inadequate to determine Cu availability as only a small proportion of ingested Cu is absorbed and differences in absorption can be masked by analytical errors.

In a 1983 symposium on the bioavailability of essential and toxic trace elements, O'Dell (1983) cited the need for the development of simplified assays for determining trace element bioavailability. Recently, Black et al. (1984a, b), working with manganese (Mn) demonstrated that tissue uptake of Mn from high level, short term supplementation of dietary Mn was a useful measure of relative bioavailability of inorganic sources. Reports of Cu accumulation in the tissues of animals fed high dietary levels of Cu (Dick, 1954; Hill and Williams, 1965; Norvell et al., 1975; Jensen and Maurice, 1979) suggest that this technique may be useful in the study of Cu bioavailability.

CHAPTER III
EFFECT OF HIGH DIETARY COPPER ON TISSUE MINERAL COMPOSITION OF BROILER AS A BIOASSAY OF CU SOURCES--EXPERIMENT 1

Introduction

Attempts to determine Cu bioavailability from inorganic sources, using traditional methods, have met with limited success (Miller, 1983). Bioassays are complicated by the facts that Cu absorption and retention values are low relative to that of other elements, and that interactions exist between Cu and other dietary constituents. In addition, Miller (1979) noted that Cu does not have an isotope with a sufficiently long half-life to permit many of the metabolic studies required.

Black et al. (1984a, b) working with manganese (Mn), found that tissue Mn accumulation during short term high level supplementation of the element can be used as a bioassay criterion in determining Mn availability from inorganic sources. This technique, described in detail by Henry et al. (1987), has several advantages over traditional techniques including the use of natural diets which are less expensive and which allow the animal to express its maximum genetic growth potential. With this technique if tissue mineral accumulation (or its transformation) is linearly

related to and correlated with dietary concentration of the element, then bioavailability can be determined by comparing test sources to a standard source using slope-ratio methods.

Reports of Cu accumulation in the tissues of chicks fed high dietary concentrations of Cu (Norvell et al., 1975; Jensen and Maurice, 1979; Robbins and Baker, 1980a, b) suggest that the technique of Black et al. (1984a, b) may be of some utility in the study of Cu bioavailability.

This experiment was conducted to determine the relationship between dietary Cu and tissue Cu accumulation and to evaluate the relative value of various tissues for use in a bioavailability assay.

Materials and Methods

One hundred and sixty-eight, day-old Cobb feather-sexed male chicks were assigned randomly to pens in a thermostatically controlled electrically heated Petersime battery with raised wire floors. Chicks were maintained on a 24 h constant-light schedule and allowed ad libitum access to feed and tap water.

A completely randomized design was used with six replicates of seven chicks assigned to each of four dietary treatments. Dietary treatments were the basal diet (Table 3-1) supplemented with 0, 100, 200 or 300 mg/kg Cu as reagent grade cupric acetate. The basal diet contained 10.5 mg/kg Cu (dry basis) by analysis and was formulated to meet the requirements of growing chicks (NRC, 1984).

TABLE 3-1. COMPOSITION OF BASAL DIET--EXPERIMENT 1

Item	%
Ingredient composition[a]	
Corn, ground yellow	55.00
Soybean meal, dehulled	37.15
Corn oil	2.50
Dicalcium phosphate	1.70
Limestone, ground	1.00
Salt, iodized	.40
DL-methionine	.25
Microingredients[b]	1.00
Corn starch	1.00
TOTAL	100.00
Chemical composition[c]	
Dry matter, %	89.3
Crude protein, %	23.0
Metabolizable energy, kcal/kg	3012.0
Ca, %	.95
P, %	.68
Mg, %	.18
Cu, mg/kg[d]	10.5
Mn, mg/kg	97
Zn, mg/kg	81
Fe, mg/kg	481

[a]As-fed basis.

[b]Ingredients supplied per kilogram of diet: Vitamin A palmitate, 6600 IU; vitamin D3, 2200 ICU; menadione dimethylpyrimidinol bisulfite, 2.2 mg; riboflavin, 4.4 mg; pantothenic acid, 13.2 mg; niacin 39.6 mg; choline chloride, 500 mg; vitamin B12, .022 mg; ethoxyquin, 125 mg; manganese, 55 mg; iron, 80 mg; copper, 4 mg; zinc, 40 mg; iodine, .35 mg.

[c]Dry matter basis. Crude protein and metabolizable energy calculated; minerals determined by analysis.

[d]Copper as $Cu[C_2H_3O_2]_2 \cdot H_2O$ added on an air dry basis at expense of corn starch.

On day 22, chicks were individually weighed and feed consumption for each pen replication was determined. Five chicks from each pen were selected randomly, killed by cervical dislocation and liver, both kidneys and right tibia excised and frozen for subsequent mineral analysis. Blood was collected by anterior cardiac puncture from the remaining two chicks and plasma saved for mineral analysis. With the exception of P, feed and tissue mineral concentrations were determined by flame atomic absorption spectrophotometry on a Perkin-Elmer Model 5000 with an AS-50 autosampler (Perkin-Elmer Corporation, 1982). Standards were matched for macroelement and acid concentrations as needed and standard reference material from the National Bureau of Standards was included with samples. Phosphorus was determined by a modified colorimetric procedure (Harris and Popat, 1954).

Data were analyzed by least square analysis of variance using the general linear models procedure of SAS (1982). In the analysis of variance, pen was used as the experimental unit for intake and feed conversion, while chick was used as the experimental unit for gain and mineral data. Duncan's multiple range test was used for mean separation. Plots of residuals from the regression of raw data indicated that liver Cu concentrations exhibited variance heterogeneity. As a result, liver Cu concentrations were subjected to log (Base 10) transformation prior to regression analysis. Adequacy of regression models were confirmed by lack of

fit analysis. Regression analysis of tissue Cu was determined by the least squares method using the general linear models procedure of SAS (1982).

Results

Analysis of chick performance (Table 3-2) indicated that dietary Cu had no effect ($P > .10$) on average daily intake, daily gain or feed conversion, which averaged 37.8 g, 24.6 g and 1.54 g/g, respectively, and were within ranges reported as normal for chicks of this age.

Liver Cu concentrations (Table 3-3) increased from 16.7 mg/kg in chicks fed the unsupplemented basal diet to 137.5 mg/kg in those fed 300 mg/kg Cu. Regression analysis of log transformed liver Cu concentrations indicated that this increase was quadratic ($P < .001$). This is illustrated in Figure 3-1, which shows the plot of the predicted equation ($\log y = 1.20 + .0000142 X + .00000197 X^2$), the actual treatment means and their associated standard errors. The regression model accounted for 70% of the variation in liver Cu.

Liver Mn concentrations were higher ($P < .05$) in chicks fed no supplemental Cu than in those fed 100 or 200 mg/kg Cu, but were similar to those fed 300 mg/kg Cu. Chicks fed 300 mg/kg Cu had higher ($P < .001$) liver P concentrations than those fed other dietary concentrations of Cu. Liver Zn, Fe, Mg and Ca concentrations were not affected ($P > .10$) by dietary Cu.

TABLE 3-2. EFFECT OF DIETARY COPPER CONCENTRATION ON FEED INTAKE, GAIN AND FEED CONVERSION IN CHICKS--EXPERIMENT 1

Added Cu, mg/kg[a]	Avg. Daily Intake, g[b]	Avg. Daily Gain, g[c]	Feed/Unit Gain[b]
0	39.4	25.6	1.54
100	37.5	24.2	1.55
200	37.1	24.4	1.52
300	37.3	24.3	1.54
SE[d]	1.0	.5	.03

[a]Basal diet contained 10.5 mg/kg Cu, by analysis.
[b]Each mean represents six pens.
[c]Each mean represents 42 chicks.
[d]Pooled standard error.

TABLE 3-3. EFFECT OF DIETARY COPPER CONCENTRATION ON MINERAL COMPOSITION OF LIVER AND KIDNEY IN CHICKS--EXPERIMENT 1

Added Cu, mg/kg[a]	Mineral Concentration (mg/kg, dry matter basis)						
	Copper	Zinc	Iron	Manganese	Magnesium	Calcium	Phosphorus
				Liver[b]			
0	16.7[c]	75.8	346	11.2[d]	747	314	9430[c]
100	16.8	76.9	356	10.3	736	315	9470
200	60.3	80.6	367	10.1	755	322	9630
300	137.5	76.5	355	10.6	750	302	9940
SE[e]	9.3	1.5	23	.3	10	6	93
				Kidney[b]			
0	14.9	73.4	300[d]	7.8[d]	827	721	12070
100	15.6	72.0	303	7.8	804	677	12390
200	16.3	74.8	280	7.3	822	705	12490
300	15.3	71.6	317	8.1	808	687	12490
SE	.5	.9	8	.2	9	19	140

[a] Basal diet contained 10.5 mg/kg Cu, by analysis.
[b] Each mean represents 30 chicks.
[c] Copper effect ($P < .001$).
[d] Copper effect ($P < .05$).
[e] Pooled standard error.

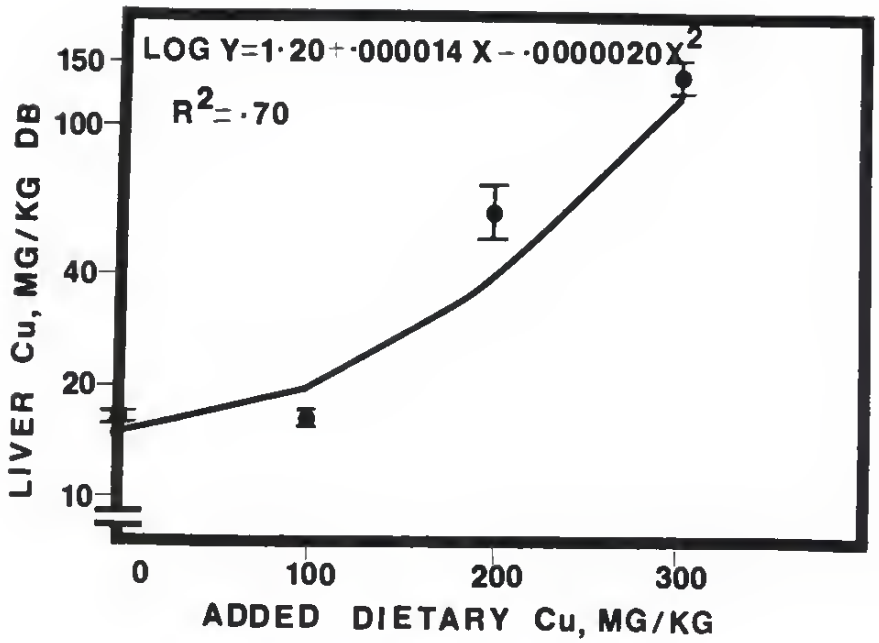

FIGURE 3-1. MEASURED AND PREDICTED COPPER CONCENTRATIONS OF LIVER WITH RESPECT TO DIETARY COPPER INTAKE IN CHICKS--EXPERIMENT 1

Dietary Cu had no effect ($P > .10$) on kidney concentrations of Cu, Zn, Mg, Ca or P (Table 3-3). Kidney Fe and Mn concentrations in chicks fed 300 mg/kg Cu were higher ($P < .05$) than in those fed 200 mg/kg Cu, but were similar to those fed 0 or 100 mg/kg Cu.

Bone ash and bone concentrations of Cu and Mg (Table 3-4) were not affected ($P > .10$) by dietary Cu. Bone Ca concentrations were highest in chicks fed no supplemental Cu, lowest in those fed 100 mg/kg Cu and intermediate in those fed 200 or 300 mg/kg Cu ($P < .001$). Chicks fed 200 mg/kg Cu had lower ($P < .01$) bone P concentrations than those fed 0 or 100 mg/kg Cu, but similar concentrations to those fed 300 mg/kg Cu.

Plasma Cu concentrations (Table 3-4, Figure 3-2) increased from 23.3 to 39.3 mg/dl as added dietary Cu increased from 0 to 300 mg/kg Cu. Regression analysis of plasma Cu concentrations indicated that the increase was linear ($P < .001$) with the regression model accounting for 35% of the variation in plasma Cu. The plot of the predicted equation ($Y = 22.4 + .0581 X$), the actual treatment means and their associated standard errors are illustrated in Figure 3-2. Plasma Ca, P, Mg, Zn and Fe concentrations were not affected ($P > .10$) by dietary Cu.

Discussion

An important requirment of the new bioassay is that mineral levels chosen do not affect animal performance

TABLE 3-4. EFFECT OF DIETARY COPPER CONCENTRATION ON BONE ASH PERCENTAGE AND MINERAL COMPOSITION OF BONE AND PLASMA IN CHICKS--EXPERIMENT 1

Added Cu, mg/kg[a]	Bone Ash, % Dry, Fat-Free Bone	Mineral Concentration					
		Calcium	Phosphorus	Magnesium	Copper	Zinc	Iron
		Bone[b]					
		%, Ash Weight Basis			mg/kg, Ash Weight Basis		
0	38.9	39.4[d]	17.3[e]	.791	3.89	---	---
100	38.9	37.7	17.3	.915	4.74	---	---
200	39.2	38.5	16.9	.839	4.92	---	---
300	39.7	38.7	17.1	.867	4.24	---	---
SE[c]	.3	.3	.1	.040	.41		
		Plasma[f]					
		mg/dl			µg/dl		
0	---	8.89	7.77	1.73	23.3	160	60.7
100	---	8.34	9.32	1.76	26.2	146	65.0
200	---	9.49	9.06	1.80	35.8	143	47.5
300	---	8.85	8.94	1.71	39.3	133	76.6
SE		.45	.45	.08	2.6	7	7.6

[a] Basal diet contained 10.5 mg/kg Cu, by analysis.
[b] Each mean represents 30 chicks.
[c] Pooled standard error.
[d] Copper effect ($P < .001$).
[e] Copper effect ($P < .01$).
[f] Each mean represents 12 chicks.

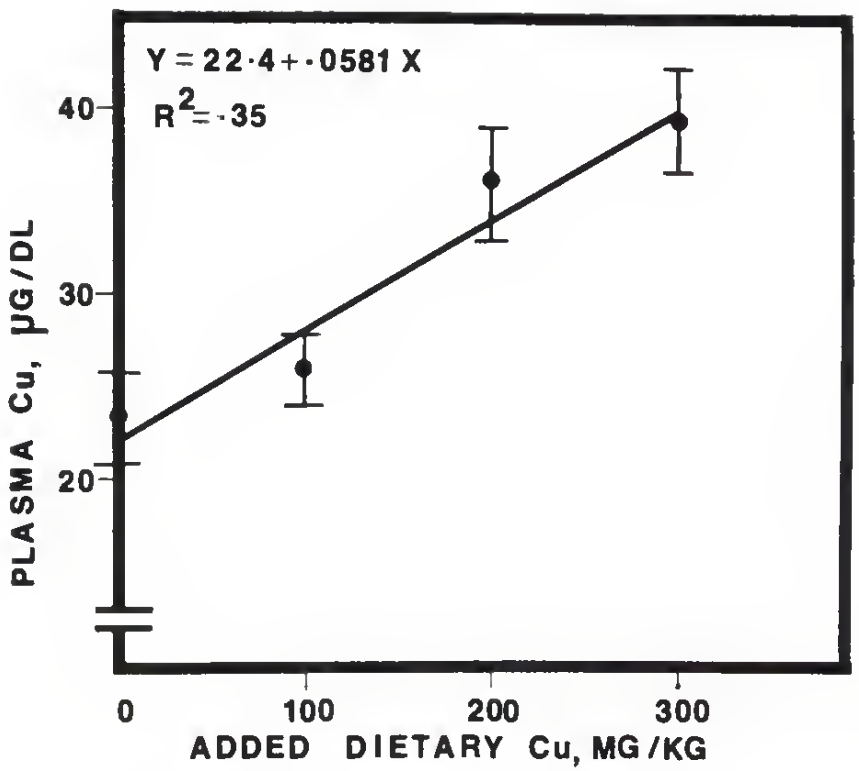

FIGURE 3-2. MEASURED AND PREDICTED COPPER CONCENTRATIONS OF PLASMA WITH RESPECT TO DIETARY COPPER INTAKE IN CHICKS--EXPERIMENT 1

(Henry et al., 1987). The highest dietary Cu concentration used in this study is the maximum tolerable level suggested for chicks by the National Research Council (1980). Chick performance was not affected by dietary Cu (as acetate) up to 300 mg/kg, indicating that those concentrations are acceptable for use in future Cu bioassays of this type and that the recommendations of the National Research Council (1980) are valid.

The primary objective of this study was to determine whether tissue Cu uptake (or its transformation) was linearly related to dietary Cu concentration. Regression analysis of plasma Cu concentrations indicated that Cu uptake by plasma was linear ($P < .001$). However, the linear model accounted for only 35% of the variation in plasma Cu and the correlation coefficient ($r = .59$) indicated a moderate linear association between plasma and dietary Cu. The moderate linear association between plasma and dietary Cu observed in this study contrasts with the very close association ($r = .99$) reported by Ekperigin and Vohra (1981b). The present study, however, differs from that of Ekperigin and Vohra (1981b) in that feeding periods were longer (22 vs 7 days) and Cu sources (acetate vs sulfate), levels (0, 100, 200 and 300 vs 0, 100, 250, 500 and 1000 mg/kg) and basal diets (corn-soybean meal vs isolated soyprotein-cornstarch) differed. These data suggest that plasma Cu accumulation may not be a useful bioassay

criterion for determining Cu bioavailability from inorganic sources.

Regression of liver Cu uptake with respect to dietary Cu indicated quadratic effects in the range measured. A graphical analysis of this response (Figure 3-1) suggests that homeostatic mechanisms in the liver of the broiler may be responsible for the quadratic effect observed. Liver Cu concentrations were unchanged as dietary Cu increased from 0 (10.5 mg/kg Cu, by analysis) to 100 mg/kg added Cu, then increased linearly from 100 to 300 mg/kg added Cu. This ability of liver to resist changes in Cu concentrations until dietary Cu concentrations exceed 100 mg/kg has been observed previously (Smith, 1969; Norvell et al., 1975; Southern and Baker, 1982) and appears to be more pronounced in broilers fed corn-soybean meal diets. The linear increase in liver Cu observed as dietary Cu increased from 100 to 300 mg/kg suggests that future bioassays should be conducted within these ranges to ensure that the tissue response falls within the linear portion of the response curve. Use of the linear portion of the curve allows bioavailability to be determined using slope-ratio methods.

The failure of dietary Cu to affect kidney Cu concentrations in this study and those of Norvell et al. (1974, 1975) and Jensen and Maurice (1979) suggest that homeostatic mechanisms in the kidney of the broiler chick are capable of maintaining kidney Cu concentrations over a wide range of dietary Cu levels. In their studies, Norvell et al. (1974,

1975) fed chicks 0, 120, 240, 480 or 720 mg/kg Cu for 8 weeks and reported no increase in kidney Cu concentrations until dietary Cu reached 720 mg/kg.

Bones were thought to be particularly responsive to changes in dietary Cu concentrations (Underwood, 1977). However, results of this study and that of Hedges and Kornegay (1973) indicate that this may not be the case as bone Cu concentrations were not affected by dietary Cu concentrations up to 60 times the requirement. A possible explanation for this contradiction is that studies cited by Underwood (1977) compared deficient bones to that of control bones whereas in this study and that of Hedges and Kornegay (1973) dietary Cu was well in excess of requirements.

Other than Cu, tissue minerals affected by dietary Cu included liver Mn and P, kidney Fe and Mn and bone Ca and P. Examination of the data, however, revealed no consistent trends either within or across tissues and although there were statistical differences, mineral concentrations were within expected ranges for broiler chicks of this age (Black, 1983; Van Ravenswaay, 1986). Data indicate that liver Cu accumulation may be a useful bioassay criterion for determining Cu bioavailability from inorganic Cu sources.

Summary

An experiment was conducted with 168 day-old male Cobb feather-sexed chicks to study the relationship between high dietary concentrations of Cu and tissue Cu accumulation.

A basal corn-soybean meal diet (10.5 mg/kg Cu) was supplemented with 0, 100, 200 or 300 mg/kg Cu as reagent grade cupric acetate ($Cu(C_2H_3O_2)_2 \cdot H_2O$) and fed ad libitum for 22 days. On day 22, chicks were killed by cervical dislocation and liver, both kidneys, bone and plasma samples collected and frozen for subsequent mineral analysis. Feed intake, gain and feed conversion were not affected ($P > .10$) by dietary treatment. Copper concentrations in liver and plasma increased ($P < .001$) with increasing dietary Cu, while that of kidney and bone remained unchanged ($P > .10$). Regression analysis indicated that the increase in plasma Cu concentrations was linear ($P < .001$) while that of liver was quadratic ($P < .001$). Other tissue minerals affected by dietary Cu included liver Mn and P, kidney Fe and Mn and bone Ca and P. Data indicate that liver Cu uptake may be a useful bioassay criterion for determining Cu bioavailability from inorganic Cu sources.

CHAPTER IV
EFFECT OF HIGH DIETARY COPPER AND AGE ON TISSUE MINERAL COMPOSITION OF BROILER CHICKS AS A BIOASSAY OF COPPER SOURCES--EXPERIMENT 2

Introduction

The distribution of body Cu among tissues varies with species, age and Cu status of the animal, but it is generally higher in liver, brain, heart and kidneys (Underwood, 1977). Tissues also differ greatly in their sensitivity to dietary Cu, with Cu concentrations in muscles, heart, endocrine glands and kidneys being relatively independent of dietary Cu, while that of liver, spleen, brain and bones is very dependent (Georgievskii, 1982). Young animals have also been shown to have higher intestinal absorption and tissue Cu concentrations than adults of the same species.

Traditional Cu bioavailability assays are complicated by Cu absorption and retention values that are low relative to that of other elements. A new bioavailability assay developed by Black et al. (1984a, b) which uses tissue mineral accumulation during short term, high level supplementation of the element as bioassay criteria, appeared to offer some utility for Cu (Exp. 1).

The fact that absorption and tissue Cu concentrations decrease with age suggests that age may significantly affect

a bioassay that is based on tissue accumulation. Therefore, the present study was conducted to determine the effect of age on tissue Cu accumulation in chicks fed high dietary concentrations of Cu.

Materials and Methods

One hundred and ninety-two, day-old Cobb feather-sexed male chicks were assigned randomly to pens in a thermostatically controlled, electrically heated Petersime battery with raised wire floors. Chicks were maintained on a 24 h constant-light schedule and allowed ad libitum access to feed and tap water.

A completely randomized design with a 4 x 3 factorial arrangement of treatments was used, with two replicate pens of eight chicks assigned to each of 12 dietary treatments. Dietary treatments included 0, 100, 200 or 300 mg/kg Cu as cupric acetate [$Cu(C_2H_3O_2)_2 \cdot H_2O$], added to the basal diet and fed for 1, 2, or 3 weeks. The basal diet (Table 4-1) contained 15.2 mg/kg Cu (dry basis) by analysis and was formulated to meet the requirements of the growing chick (NRC, 1984).

At the end of weeks 1, 2 or 3 of the experiment, chicks from the two replicates of each dietary treatment were weighed individually and feed consumption was determined for each replication. Blood was collected by anterior cardiac puncture from two chicks per replicate and plasma saved for mineral analysis. Chicks used to obtain blood samples and

TABLE 4-1. COMPOSITION OF BASAL DIET--EXPERIMENT 2

Item	%
Ingredient composition[a]	
Corn, ground yellow	55.00
Soybean meal, dehulled	37.15
Corn oil	2.50
Dicalcium phosphate	1.70
Limestone, ground	1.00
Salt, iodized	.40
DL-methionine	.25
Microingredients[b]	1.00
Corn starch	1.00
TOTAL	100.00
Chemical composition[c]	
Dry matter, %	88.8
Crude protein, %	23.0
Metabolizable energy, kcal/kg	3012.0
Ca, %	.96
P, % Total	.79
Mg, %	.20
Cu, mg/kg[d]	15.17
Mn, mg/kg	110
Zn, mg/kg	81
Fe, mg/kg	646

[a]As-fed basis.

[b]Ingredients supplied per kilogram of diet: Vitamin A palmitate, 6600 IU; vitamin D3, 2200 ICU; menadione dimethylpyrimidinol bisulfite, 2.2 mg; riboflavin, 4.4 mg; pantothenic acid, 13.2 mg; niacin 39.6 mg; choline chloride, 500 mg; vitamin B12, .022 mg; ethoxyquin, 125 mg; manganese, 55 mg; iron, 80 mg; copper, 4 mg; zinc, 40 mg; iodine, .35 mg.

[c]Dry matter basis. Crude protein and metabolizable energy calculated; dry matter and minerals determined by analysis.

[d]Copper as $Cu[C_2H_3O_2]_2 \cdot H_2O$ added on an air dry basis at expense of corn starch.

the remaining six per replicate were killed by cervical dislocation and their livers, both kidneys, pectoralis major muscles and right tibias excised and frozen for subsequent mineral analysis. With the exception of P, feed and tissue mineral concentrations were determined by flame atomic absorption spectrophotometry on a Perkin-Elmer Model 5000 with an AS-50 Autosampler (Perkin-Elmer Corporation, 1982). Standards were matched for macroelement and acid concentrations as needed and standard reference material from the National Bureau of Standards was included with samples. Phosphorus was determined by a modified colorimetric procedure (Harris and Popat, 1954).

Data were analyzed by least squares analysis of variance using the general linear models procedure of SAS (1982). The model included main effects of age and Cu level and their interaction. In the analysis of variance, pen was used as the experimental unit for intake and feed conversion, while chick was used as the experimental unit for gain and mineral data. When interactions were present, interaction means within each factor were compared separately using Duncan's multiple range test. Liver Cu concentrations exhibited variance heterogeneity and were subjected to log (Base 10) transformation prior to regression analysis. Adequacy of regression models were verified by lack of fit analysis. Regression analysis of tissue Cu was determined by the least squares method using the general linear models procedure of SAS (1982).

Results

Average daily intake, daily gain and feed conversion of chicks fed dietary treatments for 1, 2 or 3 weeks are presented in Table 4-2. There were significant ($P < .01$) age x Cu interactions observed for intake and gain in which response to dietary Cu varied with age. As expected, both intake and gain increased ($P < .001$) with age regardless of dietary Cu concentration. There was no age x Cu interaction ($P > .10$) observed for feed conversion nor was there an effect ($P > .10$) due to dietary Cu. There was, however, an effect ($P < .01$) due to age with feed to gain ratio increasing from 1.43 at week 1 to 1.55 by week 3.

Liver mineral composition as influenced by dietary Cu and age is shown in Table 4-3. A significant ($P < .001$) age x Cu interaction was observed for liver Cu. Liver Cu concentrations were not affected by dietary Cu at week 1, but increased with increasing dietary Cu at weeks 2 and 3. Regression of log transformed liver Cu on dietary Cu by age indicated that these increases were quadratic ($P < .001$). This is illustrated in Figure 4-1 which shows plots of the predicted equations, actual treatment means and standard errors of those means. The regression models accounted for 66% of the variation in liver Cu at both weeks 2 and 3. Liver Mn was not affected ($P > .10$) by dietary Cu, but did increase ($P < .001$) with age from 8.13 mg/kg at week 1 to 10.45 mg/kg by week 3. The age x Cu interaction was

TABLE 4-2. EFFECT OF DIETARY COPPER AND AGE ON FEED INTAKE, GAIN AND FEED CONVERSION IN CHICKS--EXPERIMENT 2

Response	Age, Weeks	Added Copper, mg/kg[a]					Statistics		
		0	100	200	300	Mean	Effect	P	SE[b]
Intake, g/d	1	18.8[c]	18.0	19.1	17.6	18.4[d]	Age	.0001	.3
	2	28.5	27.6	30.0	28.4	28.6	Cu	.0006	.4
	3	44.9	44.1	42.8	38.3	42.5	Age x Cu	.0013	.6
	Mean[e]	30.7	29.9	30.6	28.1	---			
Gain, g/d	1	13.5[f]	12.5	14.0	11.7	12.9[g]	Age	.0001	.3
	2	19.0	19.5	21.4	20.1	20.0	Cu	.0007	.4
	3	28.5	29.0	28.2	25.2	27.7	Age x Cu	.0064	.6
	Mean[h]	20.3	20.3	21.2	19.0	---			
Feed/Gain, g/g	1	1.39[c]	1.47	1.36	1.51	1.43[d]	Age	.0096	.02
	2	1.50	1.47	1.41	1.46	1.46	Cu	.2086	.03
	3	1.58	1.57	1.51	1.56	1.55	Age x Cu	.7441	.05
	Mean[e]	1.49	1.50	1.43	1.51	---			

[a]Basal diet contained 15.2 mg/kg Cu, by analysis.
[b]Pooled standard error.
[c]Each interaction mean represents two pens.
[d]Each main effect mean for age represents eight pens.
[e]Each main effect mean for Cu level represents six pens.
[f]Each interaction mean represents 16 chicks.
[g]Each main effect mean for age represents 64 chicks.
[h]Each main effect mean for Cu level represents 48 chicks.

TABLE 4-3. EFFECT OF DIETARY COPPER AND AGE ON THE MINERAL COMPOSITION OF LIVER IN CHICKS--EXPERIMENT 2

Mineral	Age, Weeks	Added Copper, mg/kg[a]					Statistics		
		0	100	200	300	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)							
Cu	1	19.2[d]	22.7	23.8	21.7	21.9	Age	.0001	3.3
	2	23.3	23.4	35.1	108.2	47.5	Cu	.0001	3.8
	3	18.5	18.5	20.5	82.3	34.9	Age x Cu	.0001	6.7
	Mean[e]	20.3	21.5	26.5	70.7	---			
Mn	1	8.32	8.60	8.49	7.09	8.13	Age	.0001	.23
	2	9.91	9.38	8.81	10.30	9.60	Cu	.3368	.26
	3	10.86	9.88	9.87	11.18	10.45	Age x Cu	.0146	.45
	Mean	9.70	9.29	9.06	9.52	---			
Zn	1	67.3	67.6	68.6	61.1	66.2	Age	.0001	1.3
	2	79.8	79.6	80.7	77.1	79.3	Cu	.0293	1.6
	3	82.0	72.4	78.0	73.1	76.4	Age x Cu	.5205	2.7
	Mean	76.4	73.2	75.8	70.4	---			
Fe	1	198	253	222	264	234	Age	.0001	14
	2	280	323	285	308	299	Cu	.0045	16
	3	318	321	306	452	349	Age x Cu	.1791	28
	Mean	265	299	271	342	---			

TABLE 4-3. Continued

Mineral	Age, Weeks	Added Copper, mg/kg[a]					Statistics		
		0	100	200	300	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)							
Ca	1	242[d]	274	247	200	241	Age	.0001	6
	2	311	316	309	282	304	Cu	.0108	7
	3	229	239	254	246	242	Age x Cu	.0752	13
	Mean[e]	261	276	270	243	---			
Mg	1	659	684	665	658	667	Age	.0001	9
	2	770	769	764	762	766	Cu	.2487	10
	3	781	723	721	708	733	Age x Cu	.2335	17
	Mean	736	725	717	709	---			
P	1	9410	10380	10280	10180	10060	Age	.0001	170
	2	12330	13010	12930	12690	12740	Cu	.8069	190
	3	12350	11450	11330	11400	11630	Age x Cu	.0525	330
	Mean	11360	11610	11510	11420	---			

[a] Basal diet contained 15.2 mg/kg Cu, by analysis.
[b] Each main effect mean for age represents 48 chicks.
[c] Pooled standard error.
[d] Each interaction mean represents 12 chicks.
[e] Each main effect mean for Cu level represents 36 chicks.

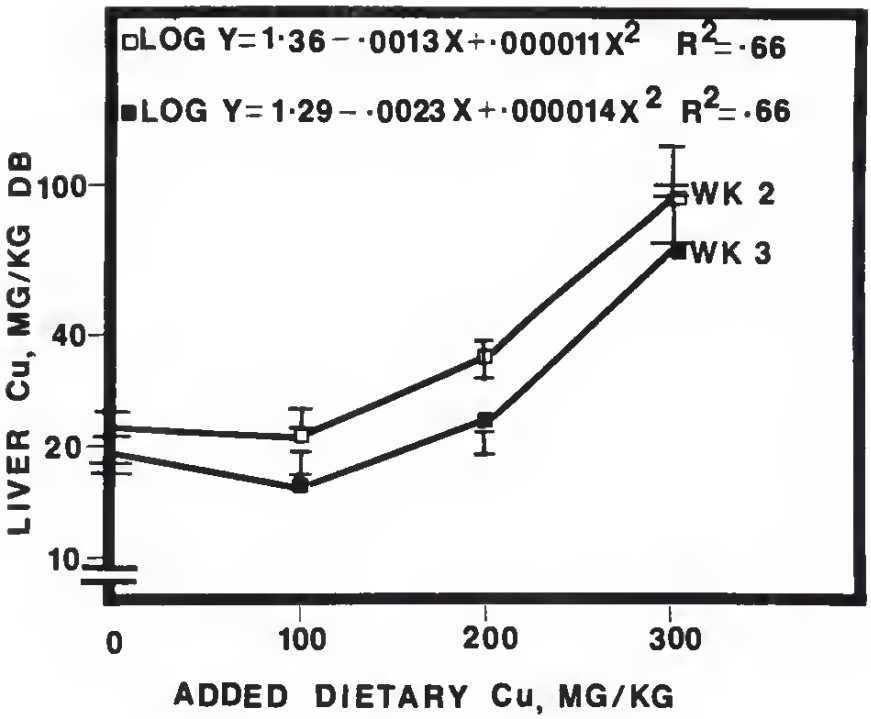

FIGURE 4-1. MEASURED AND PREDICTED COPPER CONCENTRATIONS OF LIVER WITH RESPECT TO DIETARY COPPER INTAKE IN CHICKS FED DIETS FOR 2 OR 3 WEEKS--EXPERIMENT 2

significant ($P < .05$) for liver Mn. Liver Zn was lower ($P < .001$) in birds fed 300 mg/kg Cu than in those fed 0 or 200 mg/kg Cu, but was not different from those fed 100 mg/kg Cu and was highest ($P < .05$) at 2 weeks of age. Iron in liver increased ($P < .001$) with age and was higher ($P < .01$) at 300 mg/kg Cu than at other dietary concentrations. Liver Ca was lower ($P < .001$) in birds fed 300 mg/kg Cu than in those fed 100 or 200 mg/kg Cu, but was not different from unsupplemented controls and was lower ($P < .01$) at week 2 compared to weeks 1 or 3. Liver Mg and P were highest ($P < .001$) at week 2, but were not affected ($P > .10$) by dietary Cu.

Table 4-4 shows the effects of dietary Cu and age on kidney mineral composition. Kidney Cu decreased ($P < .001$) with age but was not affected ($P > .10$) by dietary Cu. Significant ($P < .05$) age x Cu interactions were observed for kidney Mn and Fe. Kidney Zn ($P < .001$), Ca ($P < .05$) and P ($P < .001$) were affected by age but not ($P > .10$) by dietary Cu. Kidney Mg increased ($P < .05$) from 718 to 763 mg/kg as supplemental Cu increased from 0 to 300 mg/kg and decreased ($P < .05$) with age.

Effects of dietary Cu and age on the mineral composition of muscle are presented in Table 4-5. Muscle Cu, Zn, Ca, Mg and P were not affected ($P > .10$) by dietary Cu, but decreased ($P < .001$) with age. The interaction of age x Cu was significant ($P < .05$) for muscle Mn. Iron in muscle

TABLE 4-4. EFFECT OF DIETARY COPPER AND AGE ON THE MINERAL COMPOSITION OF KIDNEY IN CHICKS--EXPERIMENT 2

Mineral	Age, Weeks	Added Copper, mg/kg[a]					Statistics		
		0	100	200	300	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)							
Cu	1	26.9[d]	24.6	27.9	30.0	27.4	Age	.0001	.8
	2	24.1	23.9	22.7	21.9	23.2	Cu	.3683	1.0
	3	18.7	20.3	21.8	23.5	21.1	Age x Cu	.2445	1.7
	Mean[e]	23.3	22.9	24.1	25.1	---			
Mn	1	7.38	8.49	9.67	9.26	8.70	Age	.0110	.19
	2	7.37	8.23	7.63	8.28	7.88	Cu	.0486	.22
	3	8.53	8.44	8.50	7.51	8.25	Age x Cu	.0021	.38
	Mean	7.76	8.39	8.60	8.35	---			
Zn	1	88.4	88.2	86.3	85.2	87.0	Age	.0002	1.5
	2	79.9	83.6	78.6	73.2	78.8	Cu	.3899	1.8
	3	79.2	76.8	80.9	78.3	78.8	Age x Cu	.6398	3.1
	Mean	82.5	82.9	81.9	78.9	---			
Fe	1	234	249	280	236	250	Age	.0001	7
	2	280	276	264	276	274	Cu	.6199	8
	3	288	291	285	334	299	Age x Cu	.0313	14
	Mean	267	272	276	282	---			

TABLE 4-4. Continued

Mineral	Age, Weeks	Added Copper, mg/kg[a]					Statistics		
		0	100	200	300	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)							
Ca	1	351[d]	373	345	356	356	Age	.0402	14
	2	400	453	333	386	393	Cu	.3832	17
	3	337	331	354	346	342	Age x Cu	.3800	29
	Mean[e]	363	386	344	362	---			
Mg	1	726	777	805	806	779	Age	.0001	9
	2	716	754	731	731	733	Cu	.0242	11
	3	712	708	726	751	724	Age x Cu	.2657	19
	Mean	718	746	754	763	---			
P	1	10470	10370	9960	10000	10200	Age	.0001	140
	2	10610	10890	10600	10810	10730	Cu	.6053	160
	3	11180	11030	10930	11210	11090	Age x Cu	.8850	270
	Mean	10760	10760	10500	10670	---			

[a] [b] Basal diet contained 15.2 mg/kg Cu, by analysis.
[c] Each main effect mean for age represents 48 chicks.
[d] Pooled standard error.
[e] Each interaction mean represents 12 chicks.
Each main effect mean for Cu levels represents 36 chicks.

TABLE 4-5. EFFECT OF DIETARY COPPER AND AGE ON THE MINERAL COMPOSITION OF MUSCLE IN CHICKS--EXPERIMENT 2

Mineral	Age, Weeks	Added Copper, mg/kg[a]					Statistics		
		0	100	200	300	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)							
Cu	1	10.6[d]	11.5	10.1	11.5	10.9	Age	.0001	.6
	2	6.1	5.0	7.8	7.3	6.5	Cu	.7689	.7
	3	4.6	4.3	4.4	4.8	4.5	Age x Cu	.7533	1.2
	Mean[e]	7.1	6.9	7.4	7.9	---			
Mn	1	1.56	1.98	1.31	2.04	1.72	Age	.0001	.08
	2	.84	.97	1.42	1.21	1.11	Cu	.0686	.09
	3	.72	.77	.76	.91	.79	Age x Cu	.0332	.16
	Mean	1.04	1.24	1.17	1.39	---			
Zn	1	30.8	31.3	29.9	32.7	31.2	Age	.0001	.4
	2	22.6	23.7	23.0	22.3	22.9	Cu	.5555	.5
	3	17.8	18.0	18.2	18.7	18.2	Age x Cu	.5757	.9
	Mean	23.7	24.3	23.7	24.6	---			
Fe	1	66.9	62.9	57.2	82.8	67.5	Age	.0001	2.3
	2	39.9	43.4	42.2	40.2	41.4	Cu	.0397	2.6
	3	30.1	31.0	29.5	37.7	32.1	Age x Cu	.1134	4.6
	Mean	45.6	45.8	43.0	53.6	---			

TABLE 4-5. Continued

Mineral	Age, Weeks	Added Copper, mg/kg[a]					Statistics		
		0	100	200	300	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)							
Ca	1	194[d]	216	229	233	218	Age	.0001	10
	2	164	149	198	192	176	Cu	.4578	11
	3	161	163	142	163	157	Age x Cu	.6674	20
	Mean[e]	173	176	190	196	---			
Mg	1	1204	1228	1208	1190	1207	Age	.0001	14
	2	1153	1189	1188	1145	1169	Cu	.9056	16
	3	1052	1032	1057	1088	1058	Age x Cu	.6177	27
	Mean	1137	1150	1151	1141	---			
P	1	10800	11190	10820	10920	10930	Age	.0001	120
	2	9070	9530	8970	9090	9170	Cu	.2361	140
	3	8500	8660	8440	8730	8580	Age x Cu	.9762	240
	Mean	9460	9790	9410	9580	---			

[a]Basal diet contained 15.2 mg/kg Cu, by analysis.
[b]Each main effect mean for age represents 48 chicks.
[c]Pooled standard error.
[d]Each interaction mean represents 12 chicks.
[e]Each main effect mean for Cu level represents 36 chicks.

decreased ($P < .001$) with age, but was higher ($P < .05$) at 300 mg/kg Cu than at 0, 100 or 200 mg/kg.

Table 4-6 illustrates the effect of dietary Cu and age on bone ash and bone mineral composition. Significant ($P < .05$) age x Cu interactions were observed for bone ash and bone P. Both main effects were also significant ($P < .001$) for bone ash, but only age was significant ($P < .001$) for bone P. Bone Cu was below detection limits in 1 week chicks. There were no differences in bone Cu either as a result of dietary Cu ($P = .075$) or age ($P > .10$). Bone Mg was lower ($P < .05$) at week 2 than at weeks 1 or 3 and the interaction of age x Cu approached significance ($P = .0598$). Calcium in bone increased ($P < .001$) from 32.1% at week 1 to 33.4% at week 3.

Plasma mineral composition as influenced by dietary Cu and age is presented in Table 4-7. Plasma Cu was not affected ($P > .10$) by dietary Cu, but there was a trend towards reduction in plasma Cu ($P = .0725$) with advanced age. Plasma Zn was not affected ($P > .10$) by age or dietary Cu. A significant ($P < .01$) age x Cu interaction was observed for plasma Ca. Magnesium in plasma decreased ($P < .001$) from 2.82 mg/dl at week 1 to 2.21 mg/dl at week 3, but was not affected ($P > .10$) by dietary Cu. Plasma P was not affected ($P = .0926$) by dietary Cu, but increased ($P < .05$) from 5.79 mg/dl at week 1 to 6.33 mg/dl by week 3.

TABLE 4-6. EFFECT OF DIETARY COPPER AND AGE ON BONE ASH PERCENTAGE AND MINERAL COMPOSITION OF BONE IN CHICKS--EXPERIMENT 2

Bone Composition	Age, Weeks	Added Copper, mg/kg[a]					Statistics		
		0	100	200	300	Mean[b]	Effect	P	SE[c]
Ash, %[d]	1	39.8	38.6	40.3	38.1	39.2	Age	.0001	.3
	2	41.5	41.0	44.6	42.5	42.4	Cu	.0001	.3
	3	43.9	43.6	44.6	43.5	43.9	Age x Cu	.0448	.5
	Mean[e]	41.7	41.1	43.1	41.4	---			
Cu, mg/kg[f]	1	---[g]	---	---	---	---	Age	.8539	1.0
	2	13.6	11.5	7.2	6.2	9.6	Cu	.0754	1.4
	3	8.7	11.3	7.6	9.9	9.4	Age x Cu	.1868	1.8
	Mean	11.1	11.4	7.4	8.0	---			
Mg, %	1	.851	.887	.823	.905	.866	Age	.0243	.010
	2	.858	.820	.823	.804	.826	Cu	.1659	.012
	3	.824	.855	.833	.877	.847	Age x Cu	.0598	.020
	Mean	.844	.854	.826	.862	---			

TABLE 4-6. Continued

Bone Composition	Age, Weeks	Added Copper, mg/kg[a]					Statistics		
		0	100	200	300	Mean[b]	Effect	P	SE[c]
Ca, %[f]	1	31.7	32.3	31.9	32.3	32.1	Age	.0001	.2
	2	32.7	32.2	31.5	31.5	32.0	Cu	.1639	.2
	3	33.3	33.9	33.2	33.2	33.4	Age x Cu	.2091	.3
	Mean[e]	32.6	32.8	32.2	32.4	---			
P, %[f]	1	16.7	16.4	16.0	15.8	16.2	Age	.0001	.1
	2	16.3	15.9	16.5	16.2	16.2	Cu	.1573	.1
	3	16.9	17.1	16.9	17.1	17.0	Age x Cu	.0064	.2
	Mean	16.6	16.5	16.5	16.3	---			

[a]Basal diet contained 15.2 mg/kg Cu, by analysis.
[b]Each main effect mean for age represents 48 chicks.
[c]Pooled standard error.
[d]Each interaction mean represents 12 chicks and is expressed as a percent of dry, fat-free bone.
[e]Each main effect mean for Cu level represents 36 chicks.
[f]Each interaction mean represents 12 chicks and is expressed on an ash weight basis.
[g]Copper concentrations below detection limits.

TABLE 4-7. EFFECT OF DIETARY COPPER AND AGE ON THE MINERAL COMPOSITION OF PLASMA IN CHICKS--EXPERIMENT 2

Mineral	Age, Weeks	Added Copper, mg/kg[a]					Statistics		
		0	100	200	300	Mean[b]	Effect	P	SE[c]
Cu, µg/dl[d]	1	80.5	74.5	77.5	83.5	79.0	Age	.0725	2.7
	2	80.5	74.5	76.5	67.0	74.6	Cu	.5764	3.1
	3	73.0	69.5	68.0	66.0	69.1	Age x Cu	.7303	5.5
	Mean[e]	78.0	72.8	74.0	72.2	---			
Zn, µg/dl[d]	1	302	299	285	330	304	Age	.1303	8
	2	313	343	337	314	327	Cu	.6495	9
	3	339	335	337	284	323	Age x Cu	.0994	16
	Mean	318	326	319	309	---			
Ca, mg/dl[d]	1	12.7	11.9	12.8	12.4	12.4	Age	.0001	.1
	2	15.2	14.1	13.7	14.3	14.3	Cu	.0005	.1
	3	13.8	13.2	12.7	11.7	12.9	Age x Cu	.0055	.2
	Mean	13.9	13.0	13.1	12.8	---			

TABLE 4-7. Continued

Mineral	Age, Weeks	Added Copper, mg/kg[a]					Statistics		
		0	100	200	300	Mean[b]	Effect	P	SE[c]
Mg, mg/dl[d]	1	3.10	2.58	2.96	2.66	2.82	Age	.0002	.08
	2	2.46	2.33	2.12	2.35	2.32	Cu	.3124	.09
	3	2.23	2.19	2.21	2.21	2.21	Age x Cu	.4244	.15
	Mean[e]	2.60	2.37	2.43	2.41	---			
P, mg/dl[d]	1	5.50	5.21	6.36	6.07	5.79	Age	.0459	.14
	2	6.00	5.93	6.07	6.86	6.21	Cu	.0926	.17
	3	6.79	6.14	6.07	6.36	6.33	Age x Cu	.1434	.29
	Mean	6.10	5.76	6.17	6.43	---			

[a]Basal diet contained 15.2 mg/kg Cu, by analysis.
[b]Each main effect mean for age represents eight chicks.
[c]Pooled standard error.
[d]Each interaction mean represents two chicks.
[e]Each main effect mean for Cu level represents six chicks.

Discussion

The reduction in intake and gain observed in chicks fed 300 mg/kg Cu for 3 weeks, while contrasting with Exp. 1, was not unexpected. The National Research Council (1980) lists 300 mg/kg Cu as the maximum tolerable level for chicks; however, it is well established that dietary concentrations of Cu in excess of 250 mg/kg often result in reduced feed intake and growth of chicks (Smith, 1969; Jensen and Maurice, 1979; Robbins and Baker, 1980a, b; Christmas and Harms, 1984). This reduction in performance has been partially attributed to a deficiency of sulfur-amino acids, as Robbins and Baker (1980a, b) have demonstrated that chicks fed high dietary levels of Cu (> 250 mg/kg) have an increased sulfur-amino acid requirement. Differences between this study and Exp. 1 may well be due to sulfur-amino acid content of the diets, as dietary ingredients were not identical and although both basal diets contained the same calculated sulfur-amino acid content actual analytical values may have been different. Performance data suggest that sulfur-amino acid content of diets need to be increased in future bioassays of this type.

Of tissues evaluated, only liver proved to be sensitive to dietary Cu. The sensitivity of liver to dietary Cu is not surprising as liver is known to be the main storage organ of the body (Underwood, 1977). As illustrated in Figure 4-1, liver Cu remained relatively constant until

dietary Cu exceeded 100 mg/kg then increased with increasing dietary Cu at weeks 2 and 3. This threshold effect observed previously (Smith, 1969; Norvell et al., 1975; Southern and Baker, 1982; Exp. 1) is not unique to the chick, similar effects having been observed in rats and pigs (Milne and Weswig, 1968). The quadratic effect observed at weeks 2 and 3 in this study and in a previous experiment (Exp. 1) indicate that liver Cu uptake cannot be assumed to be linear down to the zero added Cu level (15.2 mg/kg). This suggests that future bioassays should be conducted at dietary levels that will ensure that the tissue response falls within the linear portion of the curve. Use of the linear portion of the curve allows bioavailability to be estimated using the slope ratio technique and avoids the use of a nonlinear model with its inherent complexities. The 2 week bioassay appeared to be the more sensitive of the two assays; however, both regression models accounted for 66% of the variation in liver Cu.

The lack of sensitivity to dietary Cu exhibited by kidney and muscle over the range measured is compatible with previous reports (Norvell et al., 1975; Jensen and Maurice, 1979; Exp. 1) and is in concurrence with the observations of Georgievskii (1982) that the Cu content of skeletal muscle and kidney is relatively independent of the dietary Cu concentration. In contrast, bones were thought to be particularly responsive to changes in dietary Cu (Underwood, 1977). Results of this and previous studies (Hedges and

Kornegay, 1973; Exp. 1) indicate that this may not be the case. No consistent trend is evident in response of plasma to Cu supplementation. In the present study and that of Jensen and Maurice (1979), dietary Cu concentrations up to 500 mg/kg had no effect on plasma Cu; however, in other studies (Ekperigin and Vohra, 1981b; Exp. 1) similar dietary Cu concentrations resulted in increased plasma Cu concentrations.

The effect of age on tissue Cu concentrations appear to be related to sensitivity of the tissues to dietary Cu. In kidney and muscle, tissues least sensitive to dietary Cu, Cu concentrations declined with age, while in plasma and bone, tissues thought to be relatively sensitive to dietary Cu, Cu concentrations were unaffected by age. In tissues not sensitive to dietary Cu, decreasing Cu concentrations with age may result from dilution as a consequence of tissue growth. With the exception of muscle in which all minerals including Cu decreased with age, no trends were apparent in response of tissue minerals other than Cu to advancing age. The reason for the variable response to advancing age is not known.

The response of tissue minerals other than Cu to dietary Cu concentrations, observed in the present study, is similar to that observed in Exp. 1, in that examination of data revealed no consistent trends either within or across tissues. Variability in the response of tissue minerals to dietary Cu is further demonstrated by the fact that although

tissues affected were essentially identical in both studies, different minerals were affected. Variability of this magnitude makes it difficult to attach biological significance to statistical differences.

Results of the present study suggest that liver Cu accumulation may be a useful bioassay criterion for determining Cu bioavailability and that 2 weeks is the minimum time required for such a bioassay.

Summary

An experiment was conducted with 192 day-old male Cobb feather-sexed chicks to study the relationship between high dietary concentrations of Cu and tissue Cu accumulation and to assess the influence or age on such a relationship. Chicks were allotted randomly to a 4 x 3 factorial arrangement of treatments which included a corn-soybean meal basal diet (15.2 mg/kg Cu, DM) supplemented with 0, 100, 200 or 300 mg/kg Cu as reagent grade cupric acetate [$Cu(C_2H_3O_2)_2 \cdot H_2O$] and fed for 1, 2 or 3 weeks. There were two replicates per treatment with eight chicks per replication. Chicks were housed in a Petersime battery and given ad libitum access to feed and tap water. At the end of each time period, chicks were killed by cervical dislocation and muscle, liver, both kidneys, bone and plasma samples collected and frozen for subsequent mineral analysis. Significant ($P < .01$) age x Cu interactions were observed for intake and gain. This resulted from reductions in

intake and gain in chicks fed 300 mg/kg Cu for 3 weeks. Feed to gain ratio was not affected ($P > .10$) by dietary Cu, but did increase ($P < .01$) with age. Liver Cu concentrations were not affected ($P > .10$) by dietary Cu at week 1, but increased ($P < .001$) with increasing dietary Cu at weeks 2 and 3. Regression of log transformed liver Cu on dietary Cu by age indicated that this increase was quadratic ($P < .001$) in nature. Regression models accounted for 66% of the variation in liver Cu in both weeks. Copper concentrations in tissues other than liver were not affected ($P > .10$) by dietary Cu. Other tissue minerals affected by dietary Cu included liver Zn, Fe and Ca ($P < .05$), kidney Mn and Mg ($P < .05$), muscle Fe ($P < .05$) and plasma Ca ($P < .001$). With the exception of muscle in which all minerals decreased ($P < .001$) with age, tissue mineral response to age varied with tissue and mineral within tissue. Data indicated that liver Cu accumulation may be a useful bioassay criterion for determining Cu bioavailability and that 2 weeks is the minimum time required for such an assay in chicks.

CHAPTER V
BIOLOGICAL AVAILABILITY OF COPPER FROM INORGANIC SOURCES FED AT HIGH DIETARY LEVELS TO BROILER CHICKS--EXPERIMENT 3

Introduction

The essentiality of Cu in animal nutrition and manifestations of Cu deficiency are well documented (Underwood, 1977). The requirement for Cu is relatively small and, as such, economic considerations concerning choice of a feed grade supplement may not be critical. However, the chance of a Cu deficiency occurring with resultant economic losses requires consideration of the biological availability of these sources (McNaughton et al., 1974). The biological availability of Cu sources is also critical in the selection of a source for use as a growth promotant or antimicrobial since dietary levels used approach the maximum tolerable level suggested for chicks and the chance of Cu toxicosis occurring is greatly enhanced.

Attempts to determine Cu availability from inorganic sources, using traditional methods have met with limited success (Miller, 1983). Bioassays are complicated by Cu absorption and retention values that are low relative to that of other elements and by interactions between Cu and other dietary constituents.

Results of previous experiments (Exp. 1 and 2) indicated that a new bioassay technique developed by Black et al. (1984a, b) may prove useful in determining Cu bioavailability from inorganic sources.

This experiment was conducted to determine if the new bioassay technique could be used successfully to determine Cu availability from inorganic feed grade sources of Cu.

Materials and Methods

Two hundred and eight, day-old Cobb feather-sexed male chicks were assigned randomly to pens in a thermostatically controlled electrically heated Petersime battery with raised wire floors. Chicks were maintained on a 24 h constant-light schedule and allowed ad libitum access to feed and tap water.

A completely randomized design was used with two replicate pens of eight chicks assigned to each of 13 dietary treatments. Dietary treatments included, the unsupplemented basal (zero added Cu) and the basal supplemented with 150, 300 or 450 mg/kg Cu as either reagent grade acetate (31.8% Cu), feed grade oxide (74.1% Cu), feed grade carbonate (54.6% Cu) or feed grade sulfate (25.1% Cu). The basal diet (Table 5-1) contained 11.1 mg/kg Cu (dry basis) by analysis, and was formulated to meet requirements of the growing chick (National Research Council, 1984). Final dietary Cu concentrations were confirmed by chemical analysis. Relative solubilities (Watson et al., 1970), magnetic susceptibility

TABLE 5-1. COMPOSITION OF BASAL DIET--EXPERIMENT 3

Item	%
Ingredient composition[a]	
Corn, ground yellow	55.00
Soybean meal, dehulled	37.15
Corn oil	2.50
Dicalcium phosphate	1.70
Limestone, ground	1.00
Salt, iodized	.40
DL-methionite	.45
Microingredients[b]	1.00
Corn starch	.80
TOTAL	100.00
Chemical composition[c]	
Dry matter, %	88.3
Crude protein, %	23.1
Metabolizable energy, kcal/kg	3012.0
Ca, %	.98
P, % Total	.80
Mg, %	.21
Cu, mg/kg[d]	11.1
Mn, mg/kg	92
Zn, mg/kg	82
Fe, mg/kg	565

[a] As-fed basis.

[b] Ingredients supplied per kilogram of diet: Vitamin A palmitate, 6600 IU; vitamin D3, 2200 ICU; menadione dimethylpyrimidinol bisulfite, 2.2 mg; riboflavin, 4.4 mg; pantothenic acid, 13.2 mg; niacin 39.6 mg; choline chloride, 500 mg; vitamin B12, .022 mg; ethoxyquin, 125 mg; manganese, 55 mg; iron, 80 mg; copper, 4 mg; zinc, 40 mg; iodine, .35 mg.

[c] Dry matter basis. Crude protein and metabolizable energy calculated; minerals determined by analysis.

[d] Copper as $Cu[C_2H_3O_2]_2 \cdot H_2O$ added on an air dry basis at expense of corn starch.

(Watson et al., 1971), chemical and physical characteristics of Cu sources were determined and X-ray diffraction patterns were interpreted (Table 5-2).

On day 21, chicks were weighed individually and feed consumption for each replicate pen determined. Blood was collected by anterior cardiac puncture from three chicks per replicate and plasma saved for mineral analysis. Chicks used to obtain blood samples and the remaining five per pen were killed by cervical dislocation and their liver, both kidneys and right tibia excised and frozen for subsequent mineral analysis. Copper, Mn, Zn, Fe, Ca and Mg concentrations in feed, tissue and Cu sources were determined by flame atomic absorption spectrophotometry on a Perkin-Elmer Model 5000 with an AS-50 Autosampler (Perkin-Elmer Corporation, 1982). Standards were matched for macroelement and acid concentrations as needed and standard reference material from the National Bureau of Standards was included with samples. Phosphorus was determined by a modified colorimeteric procedure (Harris and Popat, 1954).

Data were analyzed by least squares analysis of variance using the general linear models procedure of SAS (1982). The model included main effects of source, Cu level and their interaction. In the analysis of variance, pen was used as the experimental unit for intake and feed conversion, while chick was used as the experimental unit for gain and mineral data. When interactions were present, interaction means within each factor were compared separately

TABLE 5-2. CHEMICAL AND PHYSICAL CHARACTERISTICS OF COPPER SOURCES--EXPERIMENT 3

	Copper Source			
	Acetate, Reagent Grade	Oxide, Feed Grade	Sulfate, Feed Grade	Carbonate, Feed Grade
Chemical Constituents, Dry Matter Basis				
Cu, %	32.1	74.1	25.1	54.6
Ca, mg/kg	---[a]	289	---	200
P, mg/kg	---	---	---	---
Mg, mg/kg	---	2140	---	212
Zn, mg/kg	---	688	7	565
Fe, mg/kg	---	5430	76	3020
Mn, mg/kg	---	45.8	---	36.3
Particle Size, %[b]				
+30	4.0	---	.6	---
-30 + 100	78.9	.3	94.2	.3
-100	17.1	99.7	5.2	99.7

TABLE 5-2. Continued

	Copper Source			
	Acetate, Reagent Grade	Oxide, Feed Grade	Sulfate, Feed Grade	Carbonate, Feed Grade
Physical Appearance	Dark blue, fine crystals	Black, fine powder	Light blue, crystals	Light green, fine powder
Relative Solubility, %[c]				
Water	95.2	---	98.9	---
Neutral Ammonium Citrate	98.8	4.9	98.8	98.7
HCl, .4%	99.9	59.1	95.5	97.7
Citric Acid, 2%	100	8.3	100	97.9
Magnetic Susceptibility, %	---	25.2	---	5.0
Interpretation of X-Ray Patterns[d]	$Cu[C_2H_3O_2]_2 \cdot H_2O$	CuO plus minor Cu_2O	$CuSO_4 \cdot 5H_2O$	$Cu_2(OH)_2CO_3$

[a] None detected.
[b] Retained by a No.30 sieve (U.S. Bureau of Standards).
[c] Solubility in solvents at 37°C for 1 hour with constant stirring expressed as % of total mineral content.
[d] Courtesy of International Minerals and Chemical Corporation, Fertilizer Group, Noralyn, Bartow, Florida.

using Duncan's multiple range test. Liver Cu concentrations exhibited variance heterogeneity and were subjected to log (Base 10) transformation prior to regression analysis. Results of Experiments 1 and 2 and preliminary analysis of the present study indicated that liver Cu uptake could not be assumed to be linear down to the zero added Cu level. Therefore, separate linear regressions were fitted over the non-zero levels within each source according to the procedure described by Freund and Littell (1981). Relative Cu bioavailability was calculated by a slope-ratio bioassay when conditions required for a valid assay were fulfilled.

Results

Chemical and physical characteristics of mineral sources were determined and are presented in Table 5-2. Analysis of mineral sources indicated Cu concentrations of 32.1, 74.1, 25.1 and 54.6% for the acetate, oxide, sulfate and carbonate sources, respectively. No mineral contaminants were detected in the reagent grade acetate, but a comparison of the feed grade sources indicated that the oxide and carbonate sources contained a number of other minerals. The acetate and sulfate sources were almost completely soluble in water, while the oxide and carbonate sources were insoluble. The acetate, sulfate and carbonate sources had essentially the same solubility (> 95%) in neutral ammonium citrate, dilute (.4%) hydrochloric acid and dilute (2%) citric acid. The oxide was the least soluble of

the sources tested, being insoluble in water and with solubilities of 4.9, 59.1 and 8.3% in neutral ammonium citrate, hydrochloric acid and citric acid, respectively. Solubility did not appear to be related to particle size. The relatively low magnetic susceptibility observed in the oxide and carbonate sources may be related to the Fe content of these sources. Interpretation of X-ray diffraction patterns indicated a high degree of purity for the acetate, sulfate and carbonate sources, while the oxide was primarily CuO with a minor proportion Cu_2O.

Analysis of chick performance (Table 5-3) indicated that dietary Cu concentration had no effect ($P > .10$) on average daily intake, daily gain or feed conversion. There was, however, an effect due to source in which chicks fed acetate consumed less feed ($P < .01$) and had lower gains ($P < .001$) than those fed oxide, sulfate or carbonate. Feed conversion was not affected ($P > .10$) by dietary Cu concentration or source.

Liver mineral composition as influenced by dietary Cu and source is presented in Table 5-4. A significant ($P < .001$) source x level interaction was observed for liver Cu. Liver Cu was not affected by dietary Cu from the oxide source but increased with increasing dietary Cu from all other sources. Regression of log transformed liver Cu on dietary Cu by source indicated that the increase was linear ($P < .001$, $R = .88$). This is illustrated in Figure 5-1 which shows plots of the predicted equations. Liver Ca

TABLE 5-3. EFFECT OF SOURCE AND LEVEL OF DIETARY COPPER ON FEED INTAKE, GAIN AND FEED CONVERSION IN CHICKS--EXPERIMENT 3

Response	Copper Source	Added Copper, mg/kg[a]					Statistics		
		0	150	300	450	Mean	Effect	P	SE[b]
Intake, g/d	Acetate	---	38.9[c]	38.8	35.1	37.6[d]	Source (S)	.0072	.8
	Oxide	---	41.9	41.3	43.1	42.1	Level (L)	.4048	.7
	Sulfate	---	43.0	39.5	38.5	40.3	S x L	.2040	1.4
	Carbonate	---	41.0	41.6	42.6	41.7			
	Mean[e]	41.4	41.2	40.3	39.8	---			
Gain, g/d	Acetate	---	25.5[f]	25.1	23.4	24.7[g]	Source (S)	.0001	.5
	Oxide	---	28.4	28.0	26.9	27.8	Level	.1857	.5
	Sulfate	---	27.2	26.5	24.9	26.2	S x L	.6313	.9
	Carbonate	---	27.4	27.7	28.5	27.9			
	Mean[h]	27.5	27.1	26.9	26.0	---			

TABLE 5-3. Continued

Response	Copper Source	Added Copper, mg/kg[a]					Statistics		
		0	150	300	450	Mean	Effect	P	SE[b]
Feed/Gain	Acetate	---	1.53[c]	1.55	1.50	1.52[d]	Source (S)	.6237	.03
	Oxide	---	1.48	1.47	1.61	1.52	Level (L)	.6035	.02
	Sulfate	---	1.58	1.49	1.55	1.54	S x L	.3199	.04
	Carbonate	---	1.49	1.50	1.49	1.49			
	Mean[e]	1.50	1.52	1.50	1.53	---			

[a]Basal diet contained 11.1 mg/kg Cu, by analysis.
[b]Pooled standard error.
[c]Each interaction mean represents two pens.
[d]Each main effect mean for Cu source represents six pens.
[e]Each main effect mean for Cu level represents eight pens, except for the zero level which represents two pens.
[f]Each interaction mean represents 16 chicks.
[g]Each main effect mean for Cu source represents 48 chicks.
[h]Each main effect mean for Cu level represents 64 chicks, except for the zero level which represents 16 chicks.

TABLE 5-4. EFFECT OF SOURCE AND LEVEL OF DIETARY COPPER ON MINERAL COMPOSITION OF LIVER IN CHICKS--EXPERIMENT 3

Mineral	Copper Source	Added Copper, mg/kg[a]					Statistics		
		0	150	300	450	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)							
Cu	Acetate	---	20.9[d]	85.2	263.3	123.1	Source (S)	.0001	8.9
	Oxide	---	18.1	18.7	19.6	18.8	Level (L)	.0001	7.7
	Sulfate	---	22.9	93.2	263.3	126.4	S x L	.0001	15.4
	Carbonate	---	16.9	44.4	106.0	55.8			
	Mean[e]	19.4	19.7	60.4	163.1	---			
Mn	Acetate	---	11.5	12.0	11.5	11.7	Source (S)	.9282	.2
	Oxide	---	11.4	12.0	11.9	11.8	Level (L)	.5615	.2
	Sulfate	---	11.9	11.6	12.1	11.9	S x L	.7801	.4
	Carbonate	---	11.5	11.6	12.0	11.7			
	Mean	9.85	11.6	11.8	11.8	---			
Zn	Acetate	---	76.0	77.5	75.2	76.2	Source (S)	.1630	1.1
	Oxide	---	76.8	78.2	80.3	78.5	Level (L)	.3915	1.0
	Sulfate	---	77.6	80.7	73.1	77.1	S x L	.2692	2.0
	Carbonate	---	79.3	79.6	80.1	79.7			
	Mean	68.8	77.4	79.0	77.2	---			

TABLE 5-4. Continued

Mineral	Copper Source	Added Copper, mg/kg[a]					Statistics		
		0	150	300	450	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)							
Fe	Acetate	---	379[d]	373	372	375	Source (S)	.2040	18
	Oxide	---	302	338	340	327	Level (L)	.9665	15
	Sulfate	---	377	369	366	370	S x L	.9681	30
	Carbonate	---	359	337	359	352			
	Mean[e]	287	354	354	359	---			
Ca	Acetate	---	359	363	392	371	Source (S)	.0135	10
	Oxide	---	327	328	344	333	Level (L)	.0342	9
	Sulfate	---	353	396	375	375	S x L	.1833	17
	Carbonate	---	324	350	382	352			
	Mean	372	341	359	373	---			
Mg	Acetate	---	721	747	716	728	Source (S)	.1756	7
	Oxide	---	742	744	739	742	Level (L)	.0304	6
	Sulfate	---	726	751	768	748	S x L	.2250	11
	Carbonate	---	721	749	750	740			
	Mean	707	727	748	743	---			

TABLE 5-4. Continued

Mineral	Copper Source	Added Copper, mg/kg[a]					Statistics		
		0	150	300	450	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)							
P	Acetate	---	10870[d]	10440	10130	10480	Source (S)	.2621	90
	Oxide	---	10740	10450	10430	10540	Level (L)	.0788	80
	Sulfate	---	10230	10110	10560	10300	S x L	.0375	160
	Carbonate	---	10440	10460	10240	10380			
	Mean[e]	10600	10570	10370	10340	---			

[a]Basal diet contained 11.1 mg/kg Cu, by analysis.
[b]Each main effect mean for Cu source represents 48 chicks.
[c]Pooled standard error.
[d]Each interaction mean represents 16 chicks.
[e]Each main effect mean for Cu level represents 64 chicks, except for the zero level which represents 16 chicks.

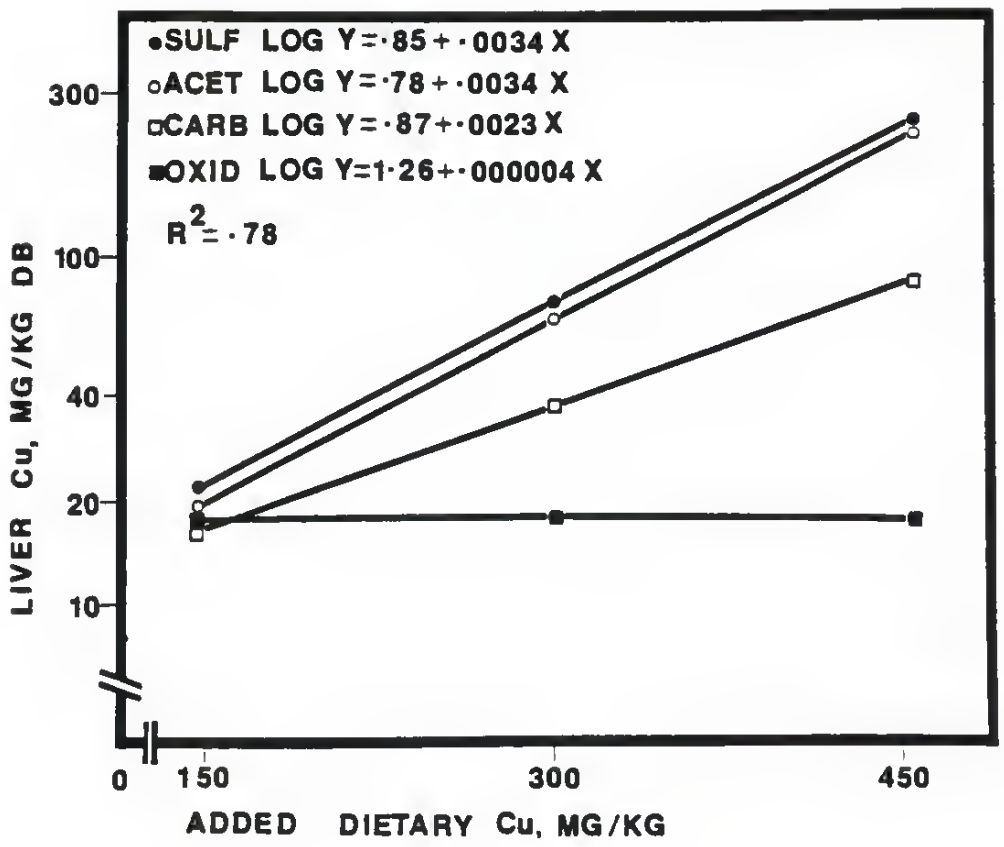

FIGURE 5-1. PREDICTED COPPER CONCENTRATIONS OF LIVER WITH RESPECT TO DIETARY COPPER INTAKE IN CHICKS--EXPERIMENT 3

increased ($P < .05$) from 341 mg/kg at 150 mg/kg Cu to 373 mg/kg at 450 mg/kg Cu and was lowest ($P < .05$) in chicks fed the oxide source. Magnesium cncentration in liver was not affected ($P > .10$) by Cu source but was lower ($P < .05$) in chicks fed 150 mg/kg Cu than in those fed 300 or 400 mg/kg Cu. A source x level interaction ($P < .05$) was observed for liver P. Liver Mn, Zn and Fe averaged 11.8, 77.9 and 356 mg/kg and were not affected ($P > .10$) by dietary treatment.

Table 5-5 summarizes the effect of dietary Cu and source on mineral composition of kidney. A source x level interaction ($P < .01$) was observed for kidney Cu, in which response to dietary Cu varied with source. Significant source x level interactions were also observed for kidney Mn ($P < .001$) and Fe ($P < .05$). Kidney P increased ($P < .001$) from 10270 to 11080 mg/kg as dietary Cu increased from 150 to 450 mg/kg, and was lowest ($P < .05$) in chicks fed acetate. Kidney Zn, Ca and Mg were not affected ($P > .10$) by dietary treatment and averaged 70.6, 432 and 739 mg/kg, respectively.

The effect of dietary Cu and source on bone ash and bone mineral composition is summarized in Table 5-6. Bone ash was not affected ($P > .10$) by dietary Cu concentration but was lower ($P < .001$) in chicks fed acetate than in those fed oxide or carbonate. Bone Cu was not influenced ($P > .10$) by Cu source but did increase ($P < .05$) from 5.3 to 6.6 mg/kg as dietary Cu concentrations increased from 150 to 300 mg/kg. Increasing dietary Cu to 450 mg/kg resulted in no

TABLE 5-5. EFFECT OF SOURCE AND LEVEL OF DIETARY COPPER ON MINERAL COMPOSITION OF KIDNEY IN CHICKS--EXPERIMENT 3

Mineral	Copper Source	Added Copper, mg/kg[a]					Statistics		
		0	150	300	450	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)							
Cu	Acetate	---	21.7[d]	18.2	24.2	21.5	Source (S)	.0032	.9
	Oxide	---	19.1	20.5	16.7	18.8	Level (L)	.4890	.8
	Sulfate	---	19.9	25.8	24.2	23.3	S x L	.0088	1.5
	Carbonate	---	22.0	21.9	22.2	22.0			
	Mean[e]	18.3	20.7	21.7	21.8	---			
Mn	Acetate	---	7.77	7.71	8.17	7.89	Source (S)	.0017	.14
	Oxide	---	8.05	8.86	8.07	8.32	Level (L)	.0699	.12
	Sulfate	---	8.51	6.92	8.28	7.91	S x L	.0001	.24
	Carbonate	---	7.25	7.44	7.95	7.54			
	Mean	7.65	7.89	7.71	8.12	---			
Zn	Acetate	---	70.7	70.8	70.8	70.8	Source (S)	.6493	.9
	Oxide	---	70.2	70.9	72.9	71.3	Level (L)	.5772	.8
	Sulfate	---	69.9	71.4	70.1	70.5	S x L	.9269	1.5
	Carbonate	---	69.3	69.0	70.9	69.7			
	Mean	67.0	70.0	70.5	71.2	---			

TABLE 5-5. Continued

Mineral	Copper Source	Added Copper, mg/kg[a]					Statistics		
		0	150	300	450	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)							
Fe	Acetate	---	265[d]	288	287	280	Source (S)	.7531	7
	Oxide	---	274	273	258	268	Level (L)	.2455	6
	Sulfate	---	294	255	274	274	S x L	.0202	13
	Carbonate	---	266	251	306	274			
	Mean[e]	254	275	266	282	---			
Ca	Acetate	---	396	369	447	405	Source (S)	.2153	19
	Oxide	---	390	460	423	425	Level (L)	.3006	16
	Sulfate	---	437	424	444	435	S x L	.6548	33
	Carbonate	---	431	459	492	462			
	Mean	400	414	430	451	---			
Mg	Acetate	---	739	756	732	742	Source (S)	.1056	7
	Oxide	---	750	751	750	750	Level (L)	.7607	6
	Sulfate	---	741	737	743	741	S x L	.3815	13
	Carbonate	---	742	702	730	725			
	Mean	709	743	736	739	---			

TABLE 5-5. Continued

Mineral	Copper Source	Added Copper, mg/kg[a]					Statistics		
		0	150	300	450	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)							
P	Acetate	---	9850[d]	10010	11110	10340	Source (S)	.0354	120
	Oxide	---	10370	10790	11090	10750	Level (L)	.0001	100
	Sulfate	---	10550	10950	10920	10800	S x L	.1622	210
	Carbonate	---	10300	10450	11190	10650			
	Mean[e]	10150	10270	10550	11080	---			

[a]Basal diet contained 11.1 mg/kg Cu, by analysis.
[b]Each main effect mean for Cu source represents 48 chicks.
[c]Pooled standard error.
[d]Each interaction mean represents 16 chicks.
[e]Each main effect mean for Cu level represents 64 chicks, except for the zero level which represents 16 chicks.

TABLE 5-6. EFFECT OF SOURCE AND LEVEL OF DIETARY COPPER ON BONE ASH PERCENTAGE AND BONE MINERAL COMPOSITION IN CHICKS--EXPERIMENT 3

Bone Composition	Copper Source	Added Copper, mg/kg[a]					Statistics		
		0	150	300	450	Mean[b]	Effect	P	SE[c]
Ash, %	Acetate	---	40.4[d]	40.8	40.6	40.6	Source (S)	.0005	.3
	Oxide	---	41.8	42.5	41.3	41.9	Level (L)	.5526	.3
	Sulfate	---	41.3	41.1	41.2	41.2	S x L	.7703	.6
	Carbonate	---	41.8	42.8	43.0	42.6			
	Mean[e]	41.8	41.3	41.8	41.5	---			
Cu, mg/kg	Acetate	---	4.5[f]	6.9	7.2	6.2	Source (S)	.8156	.4
	Oxide	---	6.6	6.3	5.9	6.2	Level (L)	.0148	.4
	Sulfate	---	5.9	6.5	7.1	6.4	S x L	.1623	.7
	Carbonate	---	4.2	6.8	6.3	5.9			
	Mean	6.0	5.3	6.6	6.6	---			
Mg, %	Acetate	---	.829[f]	.842	.863	.845	Source (S)	.0017	.007
	Oxide	---	.815	.826	.812	.818	Level (L)	.8261	.006
	Sulfate	---	.846	.845	.841	.844	S x L	.3877	.012
	Carbonate	---	.827	.802	.817	.815			
	Mean	.851	.829	.828	.833	---			

TABLE 5-6. Continued

Bone Composition	Copper Source	Added Copper, mg/kg[a]					Statistics		
		0	150	300	450	Mean[b]	Effect	P	SE[c]
Ca, %	Acetate	---	38.5[f]	38.3	38.5	38.4	Source (S)	.3165	.5
	Oxide	---	37.4	38.0	36.9	37.4	Level (L)	.8400	.4
	Sulfate	---	37.8	36.6	38.6	37.6	S x L	.6683	.8
	Carbonate	---	37.7	37.1	37.1	37.3			
	Mean[e]	38.5	37.8	37.5	37.7	---			
P, %	Acetate	---	17.2[f]	16.9	17.0	17.1	Source (S)	.7118	.1
	Oxide	---	17.1	17.3	16.8	17.1	Level (L)	.0052	.1
	Sulfate	---	16.7	17.4	17.1	17.1	S x L	.0001	.1
	Carbonate	---	16.9	17.1	17.0	17.0			
	Mean	17.3	17.0	17.2	17.0	---			

[a] Basal diet contained 11.1 mg/kg Cu, by analysis.
[b] Each main effect mean for Cu source represents 48 chicks.
[c] Pooled standard error.
[d] Each interaction mean represents 16 chicks and is expressed as a percent of dry, fat-free bone.
[e] Each main effect mean for Cu level represents 64 chicks except for the zero level which represents 16 chicks.
[f] Each interaction mean represents 16 chicks and is expressed on an ash weight basis.

further increase in bone Cu. Magnesium concentration in bone was not affected ($P > .10$) by dietary Cu concentration but was lower ($P < .10$) in chicks fed oxide and carbonate than in those fed acetate or sulfate. Bone Ca was not influenced ($P > .10$) by dietary Cu concentration or source and averaged 37.7 mg/kg. There was a source x level interaction ($P < .001$) for bone P.

Table 5-7 summarizes the influence of dietary treatment on plasma mineral composition. Copper concentrations in plasma were below detection limits. However, this may have occurred as a result of problems associated with processing of plasma samples. Significant source x level interactions were observed for plasma Zn ($P < .01$), Fe ($P < .001$) and Mg ($P < .05$). Plamsa Ca was not affected ($P > .09$) by Cu source, but decreased ($P < .001$) from 12.0 mg/dl at 150 mg/kg Cu to 11.4 mg/dl at 450 mg/kg Cu. Phosphorus concentration in plasma was not affected ($P > .08$) by dietary Cu, but was lower ($P < .01$) in chicks fed acetate than in those fed oxide, sulfate or carbonate.

Evaluation of tissues with respect to Cu uptake indicated that liver was the only tissue in which Cu uptake was linearly related to dietary Cu. The linear increase in liver Cu observed as dietary Cu increased allows its use as a bioassay criterion in a slope ratio bioassay. With the exception of oxide, all other sources fulfilled the requirement of a valid slope-ratio bioassay, namely linearity of response and a common intercept. The failure of the oxide

TABLE 5-7. EFFECT OF SOURCE AND LEVEL OF DIETARY COPPER ON MINERAL COMPOSITION OF PLASMA IN CHICKS--EXPERIMENT 3

Mineral Concentration	Copper Source	Added Copper, mg/kg[a]					Statistics		
		0	150	300	450	Mean[b]	Effect	P	SE[c]
Zn, µg/dl	Acetate	---	160[d]	177	157	165	Source (S)	.1839	6
	Oxide	---	179	167	184	177	Level (L)	.0601	5
	Sulfate	---	195	166	125	162	S x L	.0018	10
	Carbonate	---	172	178	175	175			
	Mean[e]	209	176	172	160	---			
Fe, µg/dl	Acetate	---	48.4	44.2	94.3	64.3	Source (S)	.0001	6.2
	Oxide	---	78.3	63.3	99.7	80.4	Level (L)	.0278	5.4
	Sulfate	---	108.0	129.8	124.7	120.8	S x L	.0001	10.8
	Carbonate	---	143.5	50.2	52.2	75.9			
	Mean	66.8	92.1	74.1	92.7	---			
Ca, mg/dl	Acetate	---	12.2	12.0	11.5	11.9	Source (S)	.0970	.1
	Oxide	---	12.0	11.4	11.7	11.7	Level (L)	.0003	.1
	Sulfate	---	12.2	11.7	11.2	11.7	S x L	.1299	.2
	Carbonate	---	11.6	11.6	11.4	11.5			
	Mean	12.2	12.0	11.7	11.4	---			

TABLE 5-7. Continued

Mineral Concentration	Copper Source	Added Copper, mg/kg[a]					Statistics		
		0	150	300	450	Mean[b]	Effect	P	SE[c]
Mg, mg/dl	Acetate	---	2.21[d]	2.27	2.43	2.30	Source (S)	.0464	.05
	Oxide	---	2.20	2.18	2.36	2.25	Level (L)	.9785	.04
	Sulfate	---	2.44	2.30	2.10	2.28	S x L	.0391	.09
	Carbonate	---	2.07	2.20	2.06	2.11			
	Mean[e]	2.24	2.23	2.24	2.24	---			
P, mg/dl	Acetate	---	7.48	6.89	7.13	7.17	Source (S)	.0013	.16
	Oxide	---	8.51	7.75	7.61	7.96	Level (L)	.0866	.14
	Sulfate	---	8.09	7.40	7.98	7.82	S x L	.1833	.27
	Carbonate	---	7.81	8.25	7.82	7.96			
	Mean	7.46	7.97	7.57	7.63	---			

[a]Basal diet contained 11.1 mg/kg Cu, by analysis.
[b]Each main effect mean for Cu source represents 18 chicks.
[c]Pooled standard error.
[d]Each interaction mean represents six chicks.
[e]Each main effect mean for Cu level represents 24 chicks, except for the zero level which represents six chicks.

source to fulfill the requirements necessary for a valid slope-ratio assay excludes its use in such an assay. With cupric acetate the standard source set at 100%, the relative biological availability values were determined to be 98.3 and 67.7% for the sulfate and carbonate sources, respectively (Table 5-8). Alternatively, if average increase in liver Cu concentration over basal is used for comparison, then the relative biological availability values were 103.2 and 35% for the sulfate and carbonate sources, respectively, with the oxide source found to be essentially unavailable (Table 5-8).

Discussion

Dietary concentrations of Cu in excess of 250 mg/kg often results in reduced feed intake and growth of chicks (Smith, 1969; Jensen and Maurice, 1979; Robbins and Baker, 1980a, b; Christmas and Harms, 1984; Exp. 1). However, failure of dietary Cu to affect chick performance in the present study was not unexpected since the basal diet was supplemented with .4% methionine, and it is well established that this level of supplemental methionine will completely alleviate the growth depressing effect of Cu observed in chicks fed diets containing as much as 500 mg/kg Cu (Jensen and Maurice, 1979; Ekperigin and Vohra, 1981b; Christmas and Harms, 1984). The lower intake and gain observed in chicks fed cupric acetate was independent of Cu level suggesting

TABLE 5-8. RELATIVE BIOLOGICAL AVAILABILITY OF COPPER SOURCES BASED ON LINEAR REGRESSION AND LIVER COPPER CONCENTRATION--EXPERIMENT 3

Source	Linear Regression Slope[a]	Relative Value, %	Average Liver Cu Increase[b]	Relative Value, %
		Liver		
Acetate	.00344	100.0	103.7	100.0
Oxide	---	---	.65	.63
Sulfate	.00339	98.3	107.0	103.2
Carbonate	.00233	67.7	36.3	35.0

[a]Slopes obtained from the regression of log transformed tissue Cu on dietary Cu.

[b]mg/kg, dry matter basis.

that some property of the compound, other than Cu, was affecting intake.

Results of tissue accumulation studies revealed that liver was the only tissue acceptable for use as a bioassay criterion for determining Cu bioavailability. The greater sensitivity to dietary Cu displayed by liver relative to that of other tissues, has been observed previously (Norvell et al., 1974, 1975; Jensen and Maurice, 1979; Exp. 1 and 2). With liver Cu accumulation as the response criterion, and using a slope ratio bioassay, Cu from feed grade sulfate and reagent grade acetate were almost equally available, whereas that from feed grade carbonate was approximately 68% as available (Table 5-8). The failure of the oxide source to fulfill the requirements necessary for a valid slope-ratio assay excluded its use in such an assay. Alternatively, using average increase in liver Cu concentration over basal for comparison, Cu from feed grade sulfate and reagent grade acetate were again equally available, whereas that from feed grade carbonate was only 35% as available and that of feed grade oxide was essentially unavailable (Table 5-8). Differences in bioavailability estimates obtained using the two techniques occurred as a consequence of the logarithmic transformation that was used to equalize variances. These results suggest that caution must be excercised when interpreting bioavailability estimates that are determined using slopes of regression lines when the response criterion has been subjected to log transformation. Similarly, when a

log transformation has been used to linearize a response curve, bioavailability estimates will change depending on the dietary concentrations at which such measurements are made.

Copper oxide was also found to be less available than copper sulfate by Willingham and Hill (1970), with chicks fed 10 mg/kg Cu from copper sulfate having higher hemoglobin (647 vs 4.7 gms %) and liver Cu levels (110 vs 49 mg/kg) after 21 days than those fed copper oxide. McNaughton et al. (1974) using hemoglobin content, hematocrit, liver Cu content, chick weight and mortality as response criteria found cuprous oxide to be 60% as available as copper sulfate. The relatively high availability of cuprous oxide, a source shown to be less available than cupric oxide (Leach, 1983; Klatt, 1983) contrasts with the present study. However, dietary Cu levels used by McNaughton et al. (1974) were below requirements and there is some evidence that Cu absorption is higher at low Cu doses than at higher doses (Underwood, 1977).

Based on liver Cu storage, Nesbit and Elmslie (1960) found copper oxide to be 27% as available as copper sulfate for rats fed dietary Cu levels of 250 and 500 mg/kg. Buescher et al. (1961) failed to detect differences in availability between copper carbonate, copper oxide and copper sulfate, when single doses (10 mg) of the radio-labelled sources were given orally to pigs and serum uptake and excretion of radiocopper was monitored. In contrast,

Bunch et al. (1961, 1965) fed weaning pigs 250 mg/kg supplemental Cu as copper sulfate, copper oxide or copper carbonate for 6 weeks and found liver Cu concentrations to be highest in those fed sulfate (218 mg/kg), lowest in those fed oxide (32 mg/kg) and intermediate in those fed carbonate (89 mg/kg).

The low availability of copper oxide observed in this and previous studies may be partially attributed to its low solubility. Norvell et al. (1974) reported that 97% of ingested Cu in the oxide form is excreted when fed continuously to broilers at a dietary level of 720 mg/kg, compared to 80, 78 and 78% for the acetate, chloride and sulfate salts, respectively. They concluded that copper oxide unlike acetate, chloride and sulfate is not well absorbed by broiler chicks and passes through the digestive tract. Results of the solubility tests conducted in the present study appear to support the conclusions of Norvell et al. (1974) since solvents used to determine relative solubility were selected based on their ability to simulate body fluids.

The lower availability of copper carbonate does not appear to be related to solubility since with the exception of water it was essentially as soluble as acetate and sulfate. Increased urinary excretion of Cu from copper carbonate may be partially responsible for its lower availability. Buescher et al. (1961) reported that pigs dosed orally with radiolabelled cupric oxide, cupric

carbonate or cupric sulfate excreted significantly more of the Cu dose via urine from carbonate (3.4%) than from oxide (1.0%) or sulfate (1.4%) sources.

The fact that plasma Cu concentrations were below detection limits was unexpected since normal plasma Cu concentrations were observed in previous experiments of this type (Exp. 1 and 2). There was, however, a problem with the processing of plasma samples in this study that may account for this discrepancy. During collection of samples, plasma was refrigerated prior to deproteinization and there appeared to be some protein precipitation. Since the majority of plamsa Cu is bound to the protein ceruloplasmin, Cu may have been sequestered in the precipitated protein.

An evaluation of the effect of dietary Cu and source on tissue minerals other than Cu, revealed no consistent trends either within or across tissues, indicating that the bioassay did not appear to be confounded by interactions between dietary treatments and tissue minerals. Similar trends in the response of tissue minerals to dietary Cu were observed in previous experiments (Exp. 1 and 2).

Results of the present study indicate that liver Cu uptake in chicks fed high but nontoxic dietary levels of Cu is a useful criterion for determining Cu bioavailability from inorganic sources. However, additional studies are required to determine if the same relative rankings will be observed at dietary levels closer to requirements.

Summary

An experiment was conducted with 208 day-old male Cobb feather-sexed chicks to study tissue uptake of Cu as a measure of biological availability of inorganic Cu sources. Chicks were allotted randomly to dietary treatments which included an unsupplemented basal corn-soybean meal diet (11.1 mg/kg Cu, DM) or the basal supplemented with 150, 300 or 450 mg/kg Cu as either reagent grade acetate (31.8% Cu), feed grade oxide (74.1% Cu), feed grade carbonate (54.6% Cu) or feed grade sulfate (25.1% Cu). Chicks were housed in a Petersime battery and allowed ad libitum access to feed and tap water for the 21-day experiment. Feed intake, gain and feed conversion were not affected ($P > .10$) by dietary Cu concentration. However, chicks fed acetate consumed less feed ($P < .10$) and had lower gains ($P < .001$) than those fed oxide, carbonate or basal diets. Liver Cu was not affected by dietary Cu from the oxide source but increased ($P < .001$) with increasing dietary Cu from all other sources. Bone Cu was lower ($P < .05$) in chicks fed 150 mg/kg Cu compared to other dietary levels of Cu and was not influenced ($P > .10$) by Cu source. Copper concentrations in kidney were not affected ($P > .10$) by dietary treatments. Regression analysis of liver Cu with respect to dietary Cu indicated a significant linear relationship ($R = .88$) between log transformed liver Cu concentrations and dietary Cu. Using the slope-ratio technique with acetate set at 100%, the

relative biological availability values were determined to be 98.3 and 67.7% for the sulfate and carbonate sources, respectively, with the oxide source being essentially unavailable.

CHAPTER VI
EFFECT OF HIGH DIETARY COPPER AND TIME ON TISSUE MINERAL COMPOSITION OF SHEEP AS A BIOASSAY OF COPPER SOURCES--EXPERIMENT 4

Introduction

Lo et al. (1984) cited the need for a reliable bioassay to determine Cu bioavailability since conventional balance techniques were found to be inadequate as only a small portion of Cu ingested is absorbed and differences in absorption may easily be masked by analytical error. In addition, Cu bioassays are complicated by a number of factors including interactions between Cu and other dietary constituents and the fact that Cu does not have an isotope with a sufficiently long half-life to permit many of the metabolic studies required (Miller, 1979).

Black et al. (1984a, b) working with Manganese (Mn) demonstrated that tissue uptake of Mn from high level, short term supplementation of dietary Mn was a useful measure of relative bioavailability of inorganic sources. Reports of Cu accumulation in the tissues of Cu loaded sheep (Dick, 1954; Barden and Robertson, 1962; Hill and Williams, 1965; Ishmael et al., 1971, 1972; Theil and Calvert, 1978) suggest that this technique may be useful in the study of Cu bioavailability.

Therefore, the present study was conducted to determine the relationship among time, high dietary Cu concentrations and tissue mineral composition and to evaluate the sensitivity of various tissues for use in a Cu bioavailability assay.

Materials and Methods

Twenty-eight crossbred wethers averaging 41 kg initially were used to study the effect of time and dietary Cu concentration on tissue Cu uptake and tissue mineral composition. For the duration of the experiment sheep were housed in individual raised, slotted floor pens, feed was restricted to 1200 g daily and tap water was available ad libitum. During a 10-d adjustment period all sheep were fed the basal corn-soybean meal-cottonseed hulls diet (Table 6-1) containing 9 mg/kg Cu (dry basis) by analysis and formulated to meet the nutrient requirements of sheep (NRC, 1985). At the end of the adjustment period, blood samples were obtained by jugular vein puncture from all animals and four sheep representing the unsupplemented controls were killed by exsanguination and liver, spleen, both kidneys and right metacarpus were excised and frozen for subsequent mineral analysis.

The remaining 24 sheep were then assigned randomly to a 3 x 2 factorial arrangement of treatments which included the basal diet supplemented with 15, 30 or 45 mg/kg Cu from reagent grade cupric acetate [$Cu(C_2H_3O_2)_2 \cdot H_2O$] fed for 15

TABLE 6-1. COMPOSITION OF BASAL DIET--EXPERIMENT 4

Item	%
Ingredient composition[a]	
Corn, ground yellow	58.5
Soybean meal	12.0
Cotton, seed hulls	21.0
Alfalfa, dehy meal	3.0
Corn, oil	3.0
Corn, starch	1.45
Limestone, ground	.55
Salt	---
Salt, trace mineralized[b]	.50
Vitamin, A, D[c]	+
TOTAL	100.00
Chemical composition[d]	
Dry matter, %	87.6
Crude protein, %	12.3
Crude fiber, %	11.7
Ca, %	.48
P, %	.28
Mg, %	.15
Cu, mg/kg[e]	9.24
Mn, mg/kg	15.9
Zn, mg/kg	81
Fe, mg/kg	207

[a] As-fed basis. Santoquin added at .0125% of total diet.

[b] Minerals supplied per kilogram of diet: Copper, 5 mg; iron, 100 mg; zinc, 50 mg; cobalt, 2.5 mg; iodine, 2.5 mg.

[c] Vitamin A, 2200 USP units/kg; vitamin D, 440 USP units/kg.

[d] Dry matter basis. Crude protein and fiber calculated, dry matter and minerals determined by analysis.

[e] Copper as $Cu[C_2H_3O_2]_2 \cdot H_2O$ added on an air dry basis at expense of corn starch.

or 30 days. Final dietary levels were confirmed by chemical analysis. At the end of each time period, animals were euthanized and blood and tissue samples collected as described previously.

With the exception of P, feed and tissue mineral concentrations were determined by flame atomic absorption spectrophotometry on a Perkin-Elmer Model 5000 with AS-50 autosampler (Perkin-Elmer Corporation, 1982). Standards were matched for macroelement and acid concentrations as needed and standard reference material from the National Bureau of Standards was included with samples. Phosphorus was determined by a modified colorimetric procedure (Harris and Popat, 1954). Hemoglobin was determined by a cyano-methemoglobin method (Unopette test 5857/5858; Becton-Dickinson, Rutherford, NJ) and hematocrit by a hematocrit method (Blu-tip microhematocrit capillary tubes; Sherwood Medical Industries, St. Louis, MO).

Data were analyzed by least squares analysis of variance using the general linear models procedure of SAS (1982). The model included main effects of time, Cu level, their interaction and the variable T-VS-CNT. The variable T-VS-CNT was created to compare the mean of controls to an overall mean of all other treatments and was constructed by setting T-VS-CNT = 0 for controls and T-VS-CNT = 1 otherwise (Freund and Littell, 1981).

Results

High dietary concentration of Cu did not affect diet palatability as all animals consumed the allotted 1200 g within 2-3 hours of feeding. Body weights of animals in all treatment groups increased over the course of the experiment; in sheep fed diets for 15 days the average weight increased from 41.0 to 42.0 kg, while in those fed diets for 30 days the average weight increased from 44.7 to 49.1 kg.

Hemoglobin and hematocrit (Table 6-2) were not different ($P > .09$) in sheep fed added dietary Cu, but did increase ($P < .001$) with time from 13.6 g/dl and 37.8% to 15.7 g/dl and 42.6%, respectively. Sheep fed added dietary Cu had higher ($P < .01$) hemoglobin and hematocrit values than control sheep (14.6 g/dl and 40.2% vs 12.3 g/dl and 35.1%, respectively).

Effects of dietary Cu concentration and time on mineral composition of serum are summarized in Table 6-2. In sheep fed added dietary Cu, serum Cu concentrations were not affected ($P > .10$) by dietary Cu concentration or time and averaged 120 µg/dl. This serum Cu concentration, however, was lower ($P < .01$) than that (210 µg/dl) observed in control animals suggesting that their previous diet contained more Cu than the basal diet fed during the adjustment period. Serum Zn was not affected ($P > .10$) by dietary Cu concentration or time and averaged 109 µg/dl.

TABLE 6-2. EFFECT OF DIETARY COPPER LEVEL AND TIME ON HEMOGLOBIN (Hb), HEMATOCRIT (Hct) AND MINERAL COMPOSITION OF SERUM IN SHEEP--EXPERIMENT 4

Serum Composition	Time, Days	Added Copper, mg/kg[a]					Statistics		
		0	15	30	45	Mean[b]	Effect	P	SE[c]
Hb, g/dl	0	12.3[d]	---	---	---	12.3	Time (T)	.0003	.3
	15	---	14.2	13.0	13.4	13.6	Level (L)	.0942	.4
	30	---	16.5	15.0	15.6	15.7	T x L	.9640	.6
	Mean[e]	12.3	15.4	14.0	14.5	---	T-VS-CNT[f]	.0020	---
Hct, %	0	35.1	---	---	---	35.1	Time (T)	.0005	.8
	15	---	38.3	37.0	38.0	37.8	Level (L)	.6748	1.0
	30	---	43.0	41.9	42.9	42.6	T x L	.9987	1.4
	Mean	35.1	40.6	39.4	40.4	---	T-VS-CNT	.0036	---
Cu, µg/dl	0	210	---	---	---	210	Time (T)	.3436	13
	15	---	150	122	118	130	Level (L)	.9530	16
	30	---	100	119	118	112	T x L	.4571	22
	Mean	210	125	120	118	---	T-VS-CNT	.0013	---
Zn, µg/dl	0	107	---	---	---	107	Time (T)	.4675	4
	15	---	121	102	100	107	Level (L)	.7034	5
	30	---	100	111	124	125	T x L	.0101	7
	Mean	107	111	106	112	---	T-VS-CNT	.7738	---

TABLE 6-2. Continued

Serum Composition	Time, Days	Added Copper, mg/kg[a]					Statistics		
		0	15	30	45	Mean[b]	Effect	P	SE[c]
Ca, mg/dl	0	9.7[d]	---	---	---	9.7	Time (T)	.0403	.1
	15	---	12.5	12.2	12.0	12.2	Level (L)	.7828	.2
	30	---	11.6	11.7	12.1	11.8	T x L	.1664	.2
	Mean[e]	9.7	12.1	11.9	12.0	---	T-VS-CNT[f]	.0001	---
Mg, mg/dl	0	3.03	---	---	---	3.03	Time (T)	.8483	.07
	15	---	2.71	2.63	2.70	2.68	Level (L)	.5269	.09
	30	---	2.78	2.71	2.50	2.66	T x L	.4667	.13
	Mean	3.03	2.74	2.67	2.60	---	T-VS-CNT	.0148	---
P, mg/dl	0	4.95	---	---	---	4.95	Time (T)	.2939	.29
	15	---	6.52	7.00	6.83	6.78	Level (L)	.7689	.36
	30	---	7.57	7.33	6.77	7.22	T x L	.5404	.50
	Mean	4.95	7.04	7.16	6.80	---	T-VS-CNT	.0011	---

[a] Basal diet contained 9.2 mg/kg Cu, by analysis.
[b] Each main effect mean for time represents 12 sheep except for the zero time which represents four sheep.
[c] Pooled standard error.
[d] Each interaction mean represents four sheep.
[e] Each main effect mean for Cu level represents eight sheep except for the zero level which represents four sheep.
[f] Compares the mean of controls to an overall mean of all other treatments.

There was, however, a time x level interaction ($P < .05$) observed for serum Zn. In sheep fed diets for 15 days, serum Zn decreased with increasing dietary Cu, while in those fed diets for 30 days serum Zn increased. Calcium in serum decreased ($P < .05$) with time from 12.2 mg/dl at day 15 to 11.8 mg/dl by day 30, but was unaffected ($P > .10$) in sheep fed added Cu. This Ca concentration (12.0 mg/dl) was higher ($P < .001$) than that (9.7 mg/dl) observed in control sheep. In sheep fed added dietary Cu, serum concentrations of Mg and P were not affected ($P > .10$) by dietary Cu concentration or time, averaging 2.67 and 7.0 mg/dl, respectively. These averages differed from controls, in that serum Mg was lower ($P < .05$, 2.67 vs 3.03 mg/dl) and serum P higher ($P < .01$, 7.00 vs 4.95 mg/dl).

Liver mineral composition as influenced by dietary Cu concentration and time is presented in Table 6-3. Liver Cu in sheep fed added dietary Cu averaged 503 mg/kg and was higher ($P < .01$) than that (332 mg/kg) of control animals. Copper concentration in livers of sheep fed added dietary Cu were not different ($P > .10$) but there was a trend ($P = .0580$) toward increased Cu storage with time. Liver Zn was not affected ($P > .10$) by dietary Cu concentration or time in sheep fed added Cu, but was higher ($P < .001$) in control animals (109 vs 80 mg/kg). Magnesium concentrations in liver were higher ($P < .001$) in control animals compared to those fed added dietary Cu (611 vs 545 mg/kg). Liver Mg also decreased ($P < .05$) with time and was higher ($P < .05$)

TABLE 6-3. EFFECT OF DIETARY COPPER LEVEL AND TIME ON THE MINERAL COMPOSITION OF LIVER IN SHEEP--EXPERIMENT 4

Mineral	Time, Days	Added Copper, mg/kg[a]					Statistics		
		0	15	30	45	Mean[b]	Effect	P	SE[c]
Cu	0	332[d]	---	---	---	322	Time (T)	.0580	24
	15	---	410	533	465	469	Level (L)	.3184	29
	30	---	524	525	561	537	T x L	.3000	41
	Mean[e]	332	467	529	513	---	T-VS-CNT[f]	.0010	---
Mn	0	7.89	---	---	---	7.89	Time (T)	.2008	.45
	15	---	7.53	8.05	7.03	7.54	Level (L)	.3478	.55
	30	---	6.51	7.43	6.16	6.70	T x L	.9658	.77
	Mean	7.89	7.02	7.74	6.60	---	T-VS-CNT	.3697	---
Zn	0	109	---	---	---	109	Time (T)	.9622	4
	15	---	73	84	82	80	Level (L)	.7757	5
	30	---	81	77	82	80	T x L	.5333	6
	Mean	109	77	81	82	---	T-VS-CNT	.0004	---
Fe	0	230	---	---	---	230	Time (T)	.3125	20
	15	---	170	209	222	199	Level (L)	.7279	24
	30	---	254	195	235	228	T x L	.3276	34
	Mean	230	210	202	229	---	T-VS-CNT	.6537	---

TABLE 6-3. Continued

Mineral	Time, Days	Added Copper, mg/kg[a]					Statistics		
		0	15	30	45	Mean[b]	Effect	P	SE[c]
Ca	0	274[d]	---	---	---	274	Time (T)	.1053	27
	15	---	373	244	198	272	Level (L)	.2251	34
	30	---	198	212	207	206	T x L	.1522	47
	Mean[e]	274	286	228	203	---	T-VS-CNT[f]	.5562	---
Mg	0	611	---	---	---	611	Time (T)	.0315	9
	15	---	546	583	550	559	Level (L)	.0268	11
	30	---	526	559	505	530	T x L	.6971	16
	Mean	611	536	571	528	---	T-VS-CNT	.0007	---
P	0	10800	---	---	---	10800	Time (T)	.0004	200
	15	---	9890	10480	9860	10080	Level (L)	.6346	240
	30	---	9040	8850	8820	8900	T x L	.5000	340
	Mean	10800	9460	9670	9340	---	T-VS-CNT	.0019	---

[a]Basal diet contained 9.2 mg/kg Cu, by analysis.
[b]Each main effect mean for time represents 12 sheep except for the zero time which represents four sheep.
[c]Pooled standard error.
[d]Each interaction mean represents four sheep.
[e]Each main effect mean for Cu level represents eight sheep except for the zero level which represents four sheep.
[f]Compares the mean of controls to an overall mean of all other treatments.

in sheep fed 30 mg/kg Cu compared to those fed 15 or 45 mg/kg Cu. In sheep fed added dietary Cu, liver P was not influenced ($P > .10$) by dietary Cu concentration, but did decrease with time from 10080 to 8900 mg/kg and was lower ($P < .01$) than that of control sheep (9490 vs 10800 mg/kg). Liver concentrations of Mn, Fe and Ca were not affected ($P > .10$) by dietary treatment or length of feeding period and averaged 7.23, 216 and 243 mg/kg, respectively.

Spleen concentrations of Cu, Mn, Fe, Ca, Mg and P (Table 6-4) were not affected ($P > .10$) by dietary treatment or time and averaged 8.88, 1.16, 2160, 283, 823 and 12730 mg/kg, respectively. Spleen Zn (Table 6-4) was numerically lower ($P = .0646$, 91 vs 99 mg/kg) in control sheep, but did not differ ($P > .10$) in sheep fed added dietary Cu for 15 or 30 days.

Copper concentrations in kidney (Table 6-5) were not affected ($P > .10$) by dietary treatments, but there was a trend ($P = .0683$) toward increased Cu storage over time. Kidney Zn (Table 6-5) was higher ($P = .0534$) in control sheep, but did not differ ($P > .10$) in sheep fed added dietary Cu for 15 or 30 days. Kidney concentrations of Mn, Fe, Ca, Mg and P (Table 6-5) were not affected ($P < .10$) by dietary treatment or time and averaged 4.00, 173, 498, 860 and 11980 mg/kg, respectively.

Table 6-6 summarizes the effects of dietary Cu concentration and time on bone ash percentage and mineral composition of bone. Bone ash was not affected ($P > .10$) by

TABLE 6-4. EFFECT OF DIETARY COPPER LEVEL AND TIME ON THE MINERAL COMPOSITION OF SPLEEN IN SHEEP--EXPERIMENT 4

Mineral	Time, Days	Added Copper, mg/kg[a]					Statistics		
		0	15	30	45	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)							
Cu	0	8.55[d]	---	---	---	8.55	Time (T)	.3418	.61
	15	---	9.11	8.18	8.26	8.52	Level (L)	.4786	.75
	30	---	9.46	8.18	10.45	9.36	T x L	.5542	1.06
	Mean[e]	8.55	9.29	8.18	9.35	---	T-VS-CNT[f]	.7350	---
Mn	0	1.20	---	---	---	1.20	Time (T)	.8419	.05
	15	---	1.31	1.09	1.09	1.16	Level (L)	.4199	.06
	30	---	1.11	1.11	1.22	1.15	T x L	.1373	.08
	Mean	1.20	1.21	1.10	1.15	---	T-VS-CNT	.5824	---
Zn	0	91	---	---	---	91	Time (T)	.7316	2
	15	---	99	102	97	100	Level (L)	.8170	3
	30	---	102	95	99	99	T x L	.3397	4
	Mean	91	100	99	98	---	T-VS-CNT	.0646	---
Fe	0	2802	---	---	---	2802	Time (T)	.4529	286
	15	---	2132	3021	1551	2234	Level (L)	.6345	350
	30	---	2234	1479	2062	1925	T x L	.1156	495
	Mean	2802	2183	2250	1807	---	T-VS-CNT	.2475	---

TABLE 6-4. Continued

Mineral	Time, Days	Added Copper, mg/kg[a]					Statistics		
		0	15	30	45	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)							
Ca	0	280[d]	---	---	---	280	Time (T)	.2383	10
	15	---	302	292	282	292	Level (L)	.6418	12
	30	---	273	257	295	275	T x L	.3074	17
	Mean[e]	280	288	274	288	---	T-VS-CNT[f]	.8669	---
Mg	0	831	---	---	---	831	Time (T)	.5276	11
	15	---	813	849	818	827	Level (L)	.6066	13
	30	---	809	809	833	817	T x L	.3376	18
	Mean	831	811	829	825	---	T-VS-CNT	.6589	---
P	0	12650	---	---	---	12650	Time (T)	.7197	210
	15	---	12750	12940	12700	12800	Level (L)	.7686	250
	30	---	12440	12630	12990	12690	T x L	.6340	360
	Mean	12650	12600	12790	12850	---	T-VS-CNT	.8159	---

[a] Basal diet contained 9.2 mg/kg Cu, by analysis.
[b] Each main effect mean for time represents 12 sheep except for the zero time which represents four sheep.
[c] Pooled standard error.
[d] Each interaction mean represents four sheep.
[e] Each main effect mean for Cu level represents eight sheep except for the zero level which represents four sheep.
[f] Compares the mean of controls to an overall mean of all other treatments.

TABLE 6-5. EFFECT OF DIETARY COPPER LEVEL AND TIME ON THE MINERAL COMPOSITION OF KIDNEY IN SHEEP--EXPERIMENT 4

Mineral	Time, Days	Added Copper, mg/kg[a]					Statistics		
		0	15	30	45	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)							
Cu	0	23.6[d]	---	---	---	23.6	Time (T)	.0683	1.2
	15	---	25.1	24.1	22.4	23.8	Level (L)	.7699	1.5
	30	---	24.1	27.9	29.4	27.1	T x L	.1866	2.1
	Mean[e]	23.6	24.6	25.9	25.9	---	T-VS-CNT[f]	.4220	---
Mn	0	3.76	---	---	---	3.76	Time (T)	.3924	.15
	15	---	4.07	4.21	4.14	4.14	Level (L)	.4918	.19
	30	---	4.24	4.03	3.59	3.95	T x L	.4074	.26
	Mean	3.76	4.15	4.12	3.86	---	T-VS-CNT	.3204	---
Zn	0	105	---	---	---	105	Time (T)	.5816	3
	15	---	95	92	91	92	Level (L)	.7772	4
	30	---	93	91	100	95	T x L	.5192	5
	Mean	105	94	92	96	---	T-VS-CNT	.0534	---
Fe	0	166	---	---	---	166	Time (T)	.8544	11
	15	---	204	158	164	175	Level (L)	.2768	14
	30	---	180	163	174	172	T x L	.6730	20
	Mean	166	192	160	169	---	T-VS-CNT	.7181	---

TABLE 6-5. Continued

Mineral	Time, Days	Added Copper, mg/kg[a]					Statistics		
		0	15	30	45	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)							
Ca	0	415[d]	---	---	---	415	Time (T)	.7364	34
	15	---	531	430	599	520	Level (L)	.3509	41
	30	---	582	510	419	504	T x L	.0728	59
	Mean[e]	415	557	470	509	---	T-VS-CNT[f]	.1400	---
Mg	0	863	---	---	---	863	Time (T)	.8141	15
	15	---	868	834	882	861	Level (L)	.6486	18
	30	---	875	862	833	857	T x L	.3142	25
	Mean	863	872	848	857	---	T-VS-CNT	.8760	---
P	0	12150	---	---	---	12150	Time (T)	.6746	180
	15	---	12000	11730	12300	12010	Level (L)	.8982	220
	30	---	11990	12010	11700	11900	T x L	.3594	310
	Mean	12150	11990	11870	12000	---	T-VS-CNT	.5599	---

[a]Basal diet contained 9.2 mg/kg Cu, by analysis.
[b]Each main effect mean for time represents 12 sheep except for the zero time which represents four sheep.
[c]Pooled standard error.
[d]Each interaction mean represents four sheep.
[e]Each main effect mean for Cu level represents eight sheep except for the zero level which represents four sheep.
[f]Compares the mean of controls to an overall mean of all other treatments.

TABLE 6-6. EFFECT OF DIETARY COPPER LEVEL AND TIME ON BONE ASH PERCENTAGE AND MINERAL COMPOSITION OF BONE IN SHEEP--EXPERIMENT 4

Bone Composition	Time, Days	Added Copper, mg/kg[a]					Statistics		
		0	15	30	45	Mean[b]	Effect	P	SE[c]
Ash, %[d]	0	64.3[e]	---	---	---	64.3	Time (T)	.6752	.3
	15	---	63.5	64.1	63.4	63.7	Level (L)	.1011	.3
	30	---	62.6	63.4	64.7	63.5	T x L	.0581	.5
	Mean[f]	64.3	63.0	63.7	64.0	---	T-VS-CNT[g]	.1880	---
Cu, mg/kg	0	2.18	---	---	---	2.18	Time (T)	.4629	.04
	15	---	2.44	2.63	2.14	2.40	Level (L)	.0002	.05
	30	---	2.46	2.41	2.19	2.35	T x L	.1483	.07
	Mean	2.18	2.45	2.52	2.17	---	T-VS-CNT	.0198	---
Ca, %[h]	0	38.0	---	---	---	38.0	Time (T)	.0532	.5
	15	---	38.1	39.2	39.1	38.8	Level (L)	.2402	.6
	30	---	36.3	37.3	38.3	37.3	T x L	.8094	.9
	Mean	38.0	37.2	38.2	38.7	---	T-VS-CNT	.9282	---

TABLE 6-6. Continued

Bone Composition	Time, Days	Added Copper, mg/kg[a]					Statistics		
		0	15	30	45	Mean[b]	Effect	P	SE[c]
Mg, %[h]	0	.740[e]	---	---	---	.740	Time (T)	.0113	.010
	15	---	.753	.770	.738	.753	Level (L)	.4039	.012
	30	---	.720	.718	.703	.713	T x L	.8278	.018
	Mean[f]	.740	.736	.744	.720	---	T-VS-CNT[g]	.7300	---
P, %	0	17.4	---	---	---	17.4	Time (T)	.0907	2
	15	---	17.5	17.9	17.6	17.7	Level (L)	.3895	3
	30	---	16.7	17.2	17.5	17.1	T x L	.5154	4
	Mean	17.4	17.1	17.6	17.6	---	T-VS-CNT	.9160	---

[a] Basal diet contained 9.2 mg/kg Cu, by analysis.
[b] Each main effect mean for time represents 12 sheep except for the zero time which represents four sheep.
[c] Pooled standard error.
[d] Expressed on a dry, fat-free basis.
[e] Each interaction mean represents four sheep.
[f] Each main effect mean for Cu level represents eight sheep except for the zero level which represents four sheep.
[g] Compares the mean of controls to an overall mean of all other treatments.
[h] Expressed on an ash weight basis.

dietary treatment or time, but the time x level interaction did approach significance ($P = .0581$). Copper concentrations in bone did not change ($P > .10$) over time, but were lower ($P < .001$) in sheep fed 45 mg/kg Cu than in those fed 15 or 30 mg/kg Cu. Bone Ca was not affected ($P > .10$) by dietary treatment but did decrease ($P = .0532$) from 38.8% at day 15 to 37.3% by day 30. Magnesium in bone also decreased ($P < .05$) over time, but was not influenced ($P > .10$) by dietary treatment. Bone P was not affected ($P > .10$) by dietary treatment or time and averaged 17.4%.

Discussion

Positive weight gains in sheep fed dietary Cu concentrations in excess of the maximum tolerable level suggested by the National Research Council (1980) have been reported previously. Dick (1954) observed no reduction in gain in sheep fed as much as 33.6 mg/kg dietary Cu for 177 days. Todd (1969) also reported no effects of high dietary Cu on live-weight gain, with animals in a state of chronic Cu toxicosis gaining at the same rate as control animals. The ability of sheep to gain weight while in a state of chronic Cu toxicosis is in direct contrast to what is observed in most species (Owen, 1981).

Increased hemoglobin levels and higher hematocrits as a result of Cu supplementation are not unexpected as Cu is known to play a role both in the mobilization of Fe for hemoglobin synthesis and in the production and maintenance

of adult red blood cells (Underwood, 1981). The failure of added dietary Cu to increase serum Cu concentrations in the present study is consistent with previous reports (Barden and Robertson, 1962; Todd and Thompson, 1963; Thompson and Todd, 1970; Ishmael et al., 1972). These researchers found that serum Cu concentrations in sheep remain normal even if the animals were in a state of chronic Cu toxicosis and did not increase until the hemolytic crisis was imminent.

Liver is known to be the main storage organ for Cu in the body (Underwood, 1977) and increased liver Cu concentrations with increased dietary Cu have been observed in many species (NRC, 1977). However, in the present study although animals fed added dietary Cu had a higher average Cu concentration than control animals, liver Cu did not increase with increasing dietary Cu. One factor that may account for this response is length of feeding period. In studies citing increased liver Cu with increasing dietary Cu, sheep were fed experimental diets for longer periods than that used in the present study (Dick, 1954; Hill and Williams, 1965; Hemingway and MacPherson, 1967). Increased liver Cu storage over time was not unexpected since unlike most species liver Cu concentrations in sheep have been shown to rise continously from birth (Underwood, 1977).

Underwood (1977) lists bone as one of many tissues that is particularly responsive to changes in dietary Cu. Results of this and previous experiments, however, suggest that this response varies with species and Cu status. At Cu

intakes below requirement, Cu supplementation resulted in increased bone Cu concentrations in rats (Lindow et al., 1929), chicks (Rucker et al., 1969), cattle and sheep (Hennig et al., 1974). However, at dietary Cu levels in excess of requirement, Cu supplementation had no effect on bone Cu concentrations in pigs (Allen and Harding, 1962; Hedges and Kornegay, 1973) or chicks (Exp. 1 and 2), but did increase bone Cu in rats (Lal and Sourkes, 1971). In the present study, the response to dietary Cu varied with Cu concentration; bone Cu concentrations in sheep fed 45 mg/kg Cu were similar to controls but lower than that of sheep fed 15 or 30 mg/kg Cu. The response of bone Cu to increasing age also appears to vary with species. In the Cu loaded rat, bone Cu has been reported to decline with age (Lal and Sourkes, 1971). A similar decline was not observed in the present study or in a previous study with chicks (Exp. 2).

Todd (1969) contends that Cu does not accumulate in kidney or spleen of sheep unless animals have undergone a hemolytic crisis. In contrast, Gopinath et al. (1974) and Gooneratne and Howell (1985) found that Cu accumulated in kidney without hemolysis. However, a consistent finding in these and other studies (Barden and Robertson, 1962; Ishmael et al., 1971, 1972; Theil and Calvert, 1978) citing elevated kidney and spleen Cu concentrations was the extremely high levels of Cu observed in livers of these animals. In all cases liver Cu concentrations were in excess of 2000 mg/kg Cu on a dry matter basis (DM), a concentration considered

symptomatic of Cu toxicosis. These data suggest that Cu is not deposited in kidney and spleen of sheep unless the animals are in a state of chronic Cu toxicosis and explains the lack of sensitivity to dietary Cu exhibited by kidney and spleen in the present study, where liver Cu concentrations averaged only 503 mg/kg (DM).

Other than Cu, tissue minerals affected by dietary treatment included serum Mg, Ca and P, liver Zn and Mg, and kidney Zn. Examination of data, however, revealed no consistent trends either within or across tissues and although there were statistical differences, mineral concentrations were within normal ranges reported in the literature (Georgievskii, 1982; Black, 1983; Van Ravenswaay, 1986). A similar response was observed in previous experiments with chicks (Exp. 1, 2 and 3). In contrast, the response to length of feeding period was consistent across tissues affected, with serum Ca, liver Mg and P, and bone Ca and Mg all declining over time. The reason why these particular minerals declined over time in these tissues is not known.

Results of the present study indicate that at dietary Cu concentrations of 15 to 45 mg/kg homeostatic mechanisms in the tissues of sheep appear capable of maintaining Cu concentrations within normal ranges for periods in excess of 30 days and as such an assay based on tissue Cu accumulation would be ineffective.

Summary

Twenty-eight Texas crossbred weather lambs weighing 41 kg initially were used to study the effects of dietary Cu concentration and time on tissue Cu accumulation and tissue mineral composition. Sheep were housed in individual raised, slotted floor pens and allowed ad libitum access to tap water. Feed intake was restricted to 1200 g daily. After a 10-day adjustment period in which all sheep were fed a basal corn-soybean meal-cottonseed hulls diet containing 8.1 mg/kg Cu (as fed basis), four sheep representing the unsupplemented controls were euthanized and tissue samples collected for analysis. The remaining 24 sheep were then allotted randomly to a 3 x 2 factorial arrangement of treatments which included the basal diet supplemented with 15, 30 or 45 mg/kg Cu from reagent grade cupric acetate [$Cu(C_2H_3O_2)_2 \cdot H_2O$] fed for 15 or 30 days. At the end of each time period, blood samples were obtained by jugular vein puncture, animals euthanized and liver, both kidneys, spleen and right metacarpus excised and frozen for mineral analysis. Hemoglobin and hematocrit were not different ($P > .09$) in sheep fed added dietary Cu, but were lower ($P < .01$) in sheep fed control diets compared to those fed added Cu (12.3 g/dl and 35.1% vs 14.6 g/dl and 40.2%, respectively). Both hemoglobin and hematocrit increased ($P < .001$) over time. Serum Cu concentrations in sheep fed added Cu were not affected ($P > .10$) by dietary Cu concentration or time,

but were lower ($P < .01$) in control animals. Copper concentration in livers of sheep fed added Cu were not affected ($P > .10$) by dietary Cu concentration, but were higher ($P > .01$) than that of controls (503 vs 332 mg/kg) and tended ($P = .0580$) to increase with time. Bone Cu did not change ($P > .10$) over time, but was lower ($P < .05$) in animals fed control diets compared to those fed added Cu (2.18 vs 2.38 mg/kg). Bone Cu was also lower ($P < .001$) in sheep fed 45 mg/kg Cu than in those fed 15 or 30 mg/kg Cu. Copper concentrations in spleen and kidney were not influenced ($P > .10$) by dietary Cu concentation or time averaging 8.88 and 25.2 mg/kg, respectively. Other tissue minerals affected by dietary Cu included serum Mg, Ca and P, liver Zn and Mg, and kidney Zn. The only tissue minerals other than Cu to be affected by length of feeding period were serum Ca, liver Mg and P, and bone Ca and Mg, all of which declined over time.

CHAPTER VII
EFFECT OF TIME AND HIGH DIETARY COPPER ON TISSUE MINERAL COMPOSITION OF SHEEP DEPLETED OF COPPER FOR THREE WEEKS--EXPERIMENT 5

Introduction

The essentiality of Cu in animal nutrition was first recognized in 1928 when Hart et al. (1928) demonstrated that Cu was required for hemoglobin regeneration in anemic rats. Within 3 years Cu deficiency in grazing cattle was demonstrated in Florida (Neal et al., 1931) and by 1939 two disorders of ruminants, neonatal ataxia and falling disease, were both recognized as Cu deficiency syndromes that could be prevented by Cu supplementation. Since then Cu deficiency among grazing ruminants has been reported worldwide (McDowell, 1985) and supplemental Cu is now provided in many areas.

Choice of a suitable supplemental source requires consideration of the biological availability of such sources. However, attempts to determine Cu bioavailability from inorganic sources, using traditional methods, have met with limited success (Miller, 1983). Bioassays are complicated by Cu absorption and retention values that are low relative to that of other elements, by interactions between Cu and other dietary constituents, and by the fact

that Cu does not have an isotope with a sufficiently long half-life to permit many of the metabolic studies required.

Previous experiments with chicks (Exp. 1, 2 and 3) indicated that a bioassay technique developed by Black et al. (1984a, b) was useful in determining Cu availability to chicks fed inorganic sources of Cu. With this technique tissue Cu accumulation during high level, short term supplementation of the element was used as bioassay criterion. Reports of Cu accumulation in the tissues of Cu loaded sheep (Dick, 1954; Barden and Robertson, 1962; Hill and Williams, 1965; Ishmael et al., 1971, 1972; Theil and Calvert, 1978) suggested that this technique may also be useful in the study of Cu bioavailability in sheep. Results of a previous study with sheep (Exp. 4), however, indicated that dietary Cu concentrations in excess of 45 mg/kg would be required before Cu accumulation may occur, since hemeostatic mechanisms in the tissues of sheep appear capable of maintaining Cu concentrations at this dietary level for periods of up to 30 days. Reports that both Cu uptake by intestinal cells and transfer to the serosal solution are elevated in Cu deficiency (Schwarz and Kirchgessner, 1974) suggest that a period of Cu depletion may also prove advantageous in promoting tissue Cu accumulation during supplementation.

The present study was conducted to determine: (1) the effect of time and high dietary levels of Cu on tissue Cu accumulation and tissue mineral composition of sheep depleted of Cu for 3 weeks by intravenous injections of

ammonium tetrathiomolybdate, and (2) to evaluate the sensitivity of various tissues for use in a Cu bioavailability assay.

Materials and Methods

Thirty mature crossbred wethers averaging 62 kg initially were used to study the effect of time and dietary Cu concentration on tissue Cu uptake and tissue mineral composition of sheep depleted of Cu for 3 weeks. Initially, all sheep were housed in two large covered floor pens and group-fed a corn-soybean meal-cottonseed hulls basal diet at a level selected to ensure an average daily intake of not more than 1200 g per animal. The basal diet (Table 7-1) contained 7.3 mg/kg Cu (dry basis) by analysis and was formulated to meet the nutrient requirements of sheep (NRC, 1985). Animals were depleted of Cu by intravenous injection of 50 mg ammonium tetrathiomolybdate (ATM) into the jugular vein twice weekly for 3 weeks.

Tetrathiomolybdate was prepared by bubbling hydrogen sulfide gas through a solution of ammonium molybdate until crystals were formed. The ammonium molybdate solution was prepared by dissolving ammonium molybdate (8.6 g) in ammonium hydroxide (106 ml) and water (24 ml). The orange crystals of ATM formed were first washed with cold distilled water followed by cold acetone then dried under a vacuum and stored at -4°C. Injectable solutions of ATM were prepared

TABLE 7-1. COMPOSITION OF BASAL DIET--EXPERIMENT 5

Item	%
Ingredient composition[a]	
Corn, ground yellow	58.5
Soybean meal	12.0
Cotton, seed hulls	21.0
Alfalfa, dehy meal	3.0
Corn, oil	3.0
Corn, starch	.95
Limestone, ground	.55
Salt	.50
Salt, trace mineralized[b]	.50
Vitamin, A, D[c]	+
TOTAL	100.00
Chemical composition[d]	
Dry matter, %	87.9
Crude protein, %	12.3
Crude fiber, %	11.7
Ca, %	.36
P, %	.31
Mg, %	.16
Cu, mg/kg[e]	7.29
Mn, mg/kg	16.5
Zn, mg/kg	81
Fe, mg/kg	235

[a]As-fed basis. Santoquin added at .0125% of total diet.

[b]Minerals supplied per kilogram of diet: Copper, 5 mg; iron, 100 mg; zinc, 50 mg; cobalt, 2.5 mg; iodine, 2.5 mg.

[c]Vitamin A, 2200 USP units/kg; vitamin D, 440 USP units/kg.

[d]Dry matter basis. Crude protein and fiber calculated, dry matter and minerals determined by analysis.

[e]Copper as $Cu[C_2H_3O_2]_2 \cdot H_2O$ added on an air dry basis at expense of corn starch.

weekly by dissolving crystals in sterile saline (.9%) to produce a 25 mg/ml solution.

Following the depletion phase, animals were placed in individual raised, slotted floor pens and fed the basal diet (1200 g/d) for five more days in order to allow animals time to clear their systems of ATM and to adapt to the pens. At the end of this adjustment period animals were assigned randomly to a 5 x 2 factorial arrangement of treatments which included the basal diet supplemented with 0, 20, 40, 60 or 80 mg/kg Cu from reagent grade cupric acetate [$Cu(C_2H_3O_2)_2 \cdot H_2O$] fed for 10 or 20 days. Dietary Cu concentrations were selected based on the results of a preliminary feeding experiment which indicated that animals of this type and size could consume diets containing as much as 225 mg/kg Cu for a period of 10 days without an adverse effect on feed intake or signs of Cu toxicosis occurring. Feed intake was restricted to 1200 g daily and tap water was available ad libitum. Final dietary levels of Cu were confirmed by chemical analysis. At the end of each time period blood samples were obtained by jugular vein puncture, animals were stunned, killed by exsanguination and liver, spleen, both kidneys and right metacarpus excised and frozen for subsequent mineral analysis. In an attempt to determine the effectiveness of the depletion technique, six sheep from the same flock with identical nutrition experience as those used in the present study were also killed and liver samples collected for analysis.

With the exception of P, feed and tissue mineral concentrations were determined by flame atomic absorption spectrophotometry on a Perkin-Elmer Model 5000 with AS-50 autosampler (Perkin-Elmer Corporation, 1982). Standards were matched for macroelement and acid concentrations as needed and standard reference material from the National Bureau of Standards was included with samples. Phosphorus was determined by a modified colorimetric procedure (Harris and Popat, 1954). Hemoglobin was determined by a cyanomethemoglobin method (Unopette Test 5857/5858; Becton-Dickinson, Rutherford, NJ) and hematocrit by a micro-hematocrit method (Blu-tip micro-hematocrit capillary tubes, Sherwood Medical Industries, St. Louis, MO).

Data were analyzed by least squares analysis of variance using the general linear models procedure of SAS (1982). The model included main effects of time, Cu level and the interaction of time and Cu level. Regression analysis of liver Cu was determined by the least squares method using the general linear models procedure of SAS (1982). Adequacy of regression models was confirmed by lack of fit analysis.

Results

Dietary concentrations of Cu did not affect diet palatability as all animals consumed the alloted 1200 g within 2-3 hours of feeding. Body weights of animals in all

treatment groups were maintained over the course of the experiment.

Hemoglobin and hematocrit (Table 7-2) were not affected ($P > .10$) by dietary Cu concentration or time, averaging 12.4 g/dl and 33.4%, respectively. Serum Zn (Table 7-2) was not affected ($P > .10$) by dietary Cu concentration but increased ($P < .001$) from 72 g/dl at day 10 to 133 g/dl by day 20. A significant ($P < .001$) time x level interaction was observed for serum Fe (Table 7-2). In sheep fed diets for 10 days, serum Fe was highest in those fed 80 mg/kg Cu and lowest in those fed 40 mg/kg Cu, while in sheep fed diets for 20 days serum Fe was highest in those fed no supplemental Cu and lowest in those fed 20 mg/kg Cu. Serum concentrations of Cu, Ca, Mg and P (Table 7-2) were not affected ($P > .10$) by dietary Cu concentration or time and averaged 86.8 µg/dl, 10.0 mg/dl, 2.32 mg/dl and 7.74 mg/dl, respectively.

Effects of dietary Cu concentration and time on the mineral composition of liver are presented in Table 7-3. A significant ($P < .01$) time x level interaction was observed for liver Cu. Liver Cu concentrations were affected ($P < .001$) by dietary Cu in both feeding periods. However, in sheep fed diets for 10 days, liver Cu concentrations increased to a maximum (598 mg/kg) at the highest dietary Cu concentration, while in sheep fed diets for 20 days, liver Cu concentration was highest (627 mg/kg) in those fed 60 mg/kg Cu. Regression of liver Cu on dietary Cu

TABLE 7-2. EFFECT OF TIME AND DIETARY COPPER LEVEL ON HEMOGLOBIN (Hb), HEMATOCRIT (Hct) AND MINERAL COMPOSITION OF SERUM IN SHEEP--EXPERIMENT 5

Serum Composition	Time, Days	Added Copper, mg/kg[a]						Statistics		
		0	20	40	60	80	Mean[b]	Effect	P	SE[c]
Hb, g/dl	10	12.5[d]	12.5	13.5	12.2	10.8	12.3	Time (T)	.9143	.6
	20	10.8	11.9	13.8	12.8	12.6	12.4	Level (L)	.6215	1.0
	Mean[e]	11.7	12.2	13.7	12.5	11.7	---	T x L	.7797	1.4
Hct, %	10	34.3	32.6	36.9	31.8	29.2	32.9	Time (T)	.6951	1.6
	20	32.2	33.1	38.0	33.5	32.5	33.9	Level (L)	.4975	2.6
	Mean	33.2	32.9	37.4	32.6	30.9	---	T x L	.9621	3.7
Cu, µg/dl	10	89	112	86	95	83	93	Time (T)	.1516	6
	20	74	77	102	81	69	81	Level (L)	.5636	9
	Mean	82	95	94	88	76	---	T x L	.4334	13
Zn, µg/dl	10	87	107	61	53	52	72	Time (T)	.0002	9
	20	159	103	156	124	124	133	Level (L)	.4335	15
	Mean	123	105	109	88	88	---	T x L	.2030	21
Fe, µg/dl	10	149	119	77	234	339	184	Time (T)	.3021	17
	20	316	92	144	137	106	159	Level (L)	.0043	26
	Mean	233	106	111	186	223	---	T x L	.0003	37

TABLE 7-2. Continued

Serum Composition	Time, Days	Added Copper, mg/kg[a]						Statistics		
		0	20	40	60	80	Mean[b]	Effect	P	SE[c]
Ca, mg/dl	10	10.4[d]	9.7	10.1	9.8	9.7	9.95	Time (T)	.3669	.1
	20	10.4	10.2	9.9	9.8	10.4	10.14	Level (L)	.4395	.2
	Mean[e]	10.4	9.9	10.0	9.8	10.0	---	T x L	.5332	.3
Mg, mg/dl	10	2.33	2.25	2.30	2.33	2.21	2.28	Time (T)	.2892	.05
	20	2.26	2.38	2.47	2.43	2.28	2.37	Level (L)	.7526	.09
	Mean	2.30	2.31	2.38	2.38	2.25	---	T x L	.8862	.12
P, mg/dl	10	8.18	8.82	6.26	7.26	7.13	7.53	Time (T)	.4784	.41
	20	7.55	7.69	7.43	8.64	8.45	7.95	Level (L)	.6216	.65
	Mean	7.87	8.26	6.85	7.95	7.79	---	T x L	.5035	.92

[a]Basal diet contained 7.3 mg/kg Cu, by analysis.
[b]Each main effect mean for time represents 15 sheep.
[c]Pooled standard error.
[d]Each interaction mean represents three sheep.
[e]Each main effect mean for Cu level represents six sheep.

TABLE 7-3. EFFECT OF TIME AND DIETARY COPPER LEVEL ON THE MINERAL COMPOSITION OF LIVER IN SHEEP--EXPERIMENT 5

Mineral	Time, Days	Added Copper, mg/kg[a]						Statistics		
		0	20	40	60	80	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)								
Cu	10	268[d]	240	444	416	598	393	Time (T)	.0409	26
	20	223	547	526	627	440	473	Level (L)	.0004	41
	Mean[e]	245	394	485	522	519	---	T x L	.0045	58
Mn	10	8.36	9.69	9.46	10.46	10.93	9.78	Time (T)	.0059	.42
	20	7.71	7.80	7.75	9.59	6.96	7.96	Level (L)	.3323	.66
	Mean	8.03	8.75	8.61	10.02	8.94	---	T x L	.4348	.93
Zn	10	110	106	126	105	106	111	Time (T)	.0033	4
	20	91	89	89	99	80	90	Level (L)	.6704	7
	Mean	101	97	108	102	93	---	T x L	.6083	10
Fe	10	249	267	258	298	173	249	Time (T)	.1042	19
	20	242	178	204	203	185	202	Level (L)	.5073	31
	Mean	245	223	231	250	179	---	T x L	.6638	43

TABLE 7-3. Continued

Mineral	Time, Days	Added Copper, mg/kg[a]						Statistics		
		0	20	40	60	80	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)								
Ca	10	128[d]	148	147	153	172	150	Time (T)	.0498	4
	20	141	140	145	141	117	137	Level (L)	.7109	7
	Mean[e]	135	144	146	147	145	---	T x L	.0243	10
Mg	10	539	632	599	609	679	612	Time (T)	.0002	14
	20	528	513	540	538	491	522	Level (L)	.5259	22
	Mean	533	572	569	574	585	---	T x L	.0834	31
P	10	10120	10270	10580	10880	11320	10640	Time (T)	.0009	280
	20	8780	9050	9180	9270	9150	9090	Level (L)	.7359	440
	Mean	9450	9660	9880	10080	10240	---	T x L	.9461	630

[a] Basal diet contained 7.3 mg/kg Cu, by analysis.
[b] Each main effect mean for time represents 15 sheep.
[c] Pooled standard error.
[d] Each interaction mean represents three sheep.
[e] Each main effect mean for Cu level represents six sheep.

by time indicated that the increase in liver Cu observed in sheep fed diets for 10 days was linear ($P < .01$) while that observed in sheep fed diets for 20 days was quadratic ($P < .01$). This is illustrated in Figures 7-1 and 7-2, which shows the plots of the predicted equations, actual treatment means and standard errors of those means. The linear model ($Y = 226 + 4.18X$) accounted for 57% of the variation in liver Cu at day 10, while the quadratic model ($Y = 241 + 15.4X - .161 X^2$) accounted for 69% of the variation of liver Cu at day 20. Liver concentrations of Mn, Zn, Mg and P were not affected ($P > .10$) by dietary Cu, but decreased ($P < .01$) with time. A significant ($P < .05$) time x level interaction was observed for liver Ca. In sheep fed diets for 10 days liver Ca increased numerically with increasing dietary Cu, while in sheep fed diets for 20 days liver Ca remained relatively constant as dietary Cu increased from 0 to 60 mg/kg, then declined numerically at 80 mg/kg dietary Cu. Liver Fe was not affected ($P > .10$) by dietary Cu or time and averaged 226 mg/kg.

Table 7-4 summarizes the effects of dietary Cu concentration and time on spleen mineral composition. Spleen concentrations of Cu, Mn, Zn, Fe, Ca, Mg and P were not affected ($P > .10$) by dietary Cu or time and averaged 6.89, 1.13, 98, 2743, 176, 792 and 12890 mg/kg, respectively.

Kidney mineral composition as influenced by dietary Cu concentration and time is shown in Table 7-5. Kidney concentrations of Cu, Zn, Fe and Ca were not influenced ($P >$

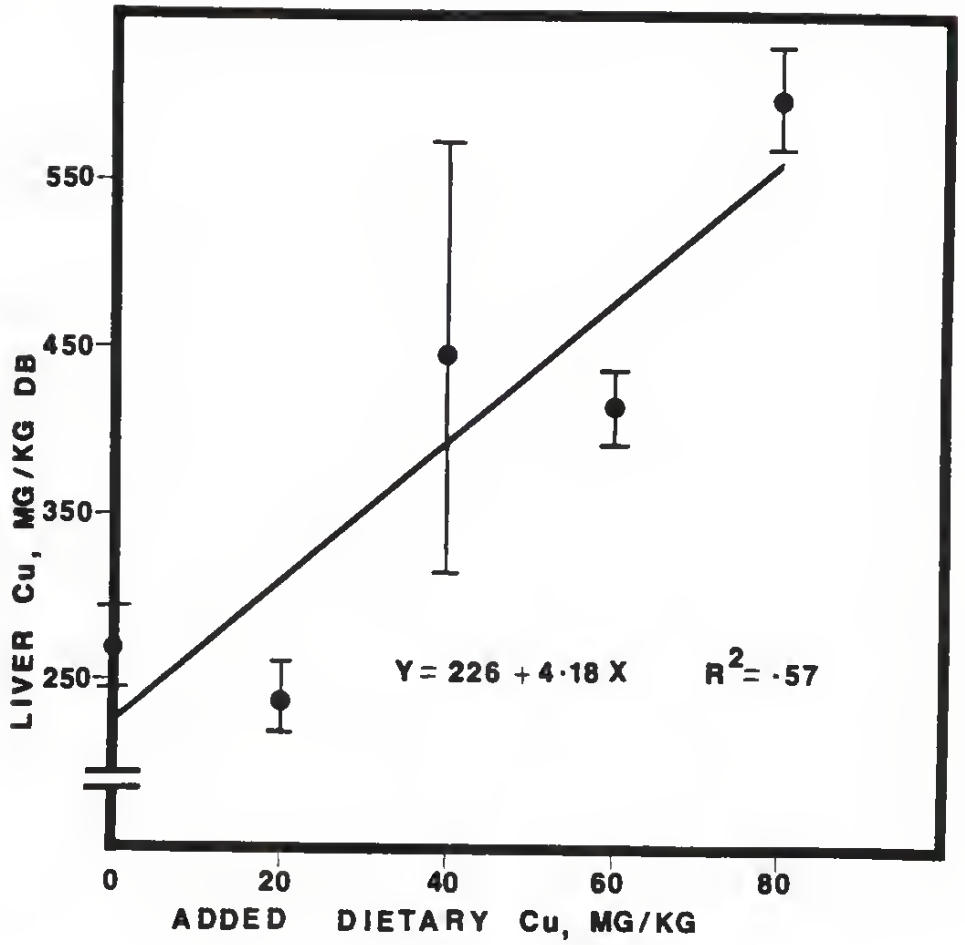

FIGURE 7-1. MEASURED AND PREDICTED COPPER CONCENTRATIONS OF LIVER WITH RESPECT TO DIETARY COPPER INTAKE IN SHEEP FED DIETS FOR 10 DAYS--EXPERIMENT 5

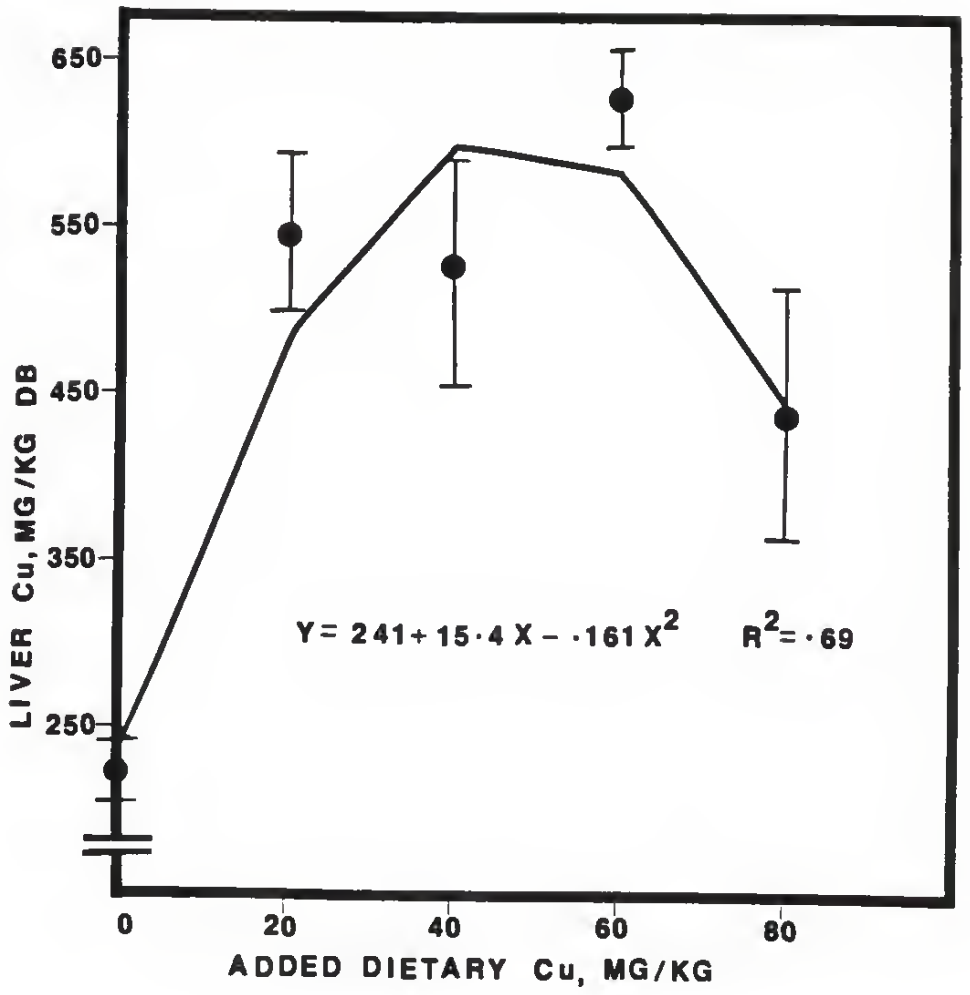

FIGURE 7-2. MEASURED AND PREDICTED COPPER CONCENTRATIONS OF LIVER WITH RESPECT TO DIETARY COPPER INTAKE IN SHEEP FED DIETS FOR 20 DAYS--EXPERIMENT 5

TABLE 7-4. EFFECT OF TIME AND DIETARY COPPER LEVEL ON THE MINERAL COMPOSITION OF SPLEEN IN SHEEP--EXPERIMENT 5

Mineral	Time, Days	Added Copper, mg/kg[a]						Statistics		
		0	20	40	60	80	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)								
Cu	10	6.73[d]	7.63	6.50	6.86	7.00	6.94	Time (T)	.7949	.30
	20	7.67	6.61	7.36	6.44	6.10	6.83	Level (L)	.8295	.47
	Mean[e]	7.20	7.12	6.93	6.65	6.55	---	T x L	.4239	.66
Mn	10	1.12	1.17	1.01	1.10	1.33	1.15	Time (T)	.5052	.04
	20	1.01	1.02	1.31	1.12	1.08	1.11	Level (L)	.5978	.07
	Mean	1.06	1.09	1.16	1.11	1.20	---	T x L	.0716	.09
Zn	10	101	107	112	94	95	102	Time (T)	.2500	4
	20	95	101	97	94	87	95	Level (L)	.5104	7
	Mean	98	104	105	94	91	---	T x L	.9477	9
Fe	10	2594	3735	2003	2525	1225	2416	Time (T)	.4019	540
	20	4239	2930	2955	2185	3044	3071	Level (L)	.7524	854
	Mean	3416	3333	2479	2355	2134	---	T x L	.7538	1208

TABLE 7-4. Continued

Mineral	Time, Days	Added Copper, mg/kg[a]						Statistics		
		0	20	40	60	80	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)								
Ca	10	178[d]	186	200	168	185	183	Time (T)	.1291	6
	20	185	159	175	166	160	169	Level (L)	.6274	10
	Mean[e]	181	173	188	167	172	---	T x L	.6571	14
Mg	10	834	842	786	755	772	798	Time (T)	.6435	17
	20	825	798	811	763	736	787	Level (L)	.1830	26
	Mean	830	820	799	759	754	---	T x L	.8686	37
P	10	13740	13980	12400	11987	12550	12930	Time (T)	.8453	290
	20	13430	13350	13290	12310	11875	12850	Level (L)	.0810	460
	Mean	13590	13660	12840	12150	12210	---	T x L	.7144	660

[a] Basal diet contained 7.3 mg/kg Cu, by analysis.
[b] Each main effect mean for time represents 15 sheep.
[c] Pooled standard error.
[d] Each interaction mean represents three sheep.
[e] Each main effect mean for Cu level represents six sheep.

TABLE 7-5. EFFECT OF TIME AND DIETARY COPPER LEVEL ON THE MINERAL COMPOSITION OF KIDNEY IN SHEEP--EXPERIMENT 5

Mineral	Time, Days	Added Copper, mg/kg[a]						Statistics		
		0	20	40	60	80	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)								
Cu	10	33.6[d]	33.6	42.3	46.9	55.1	42.3	Time (T)	.4541	2.5
	20	41.0	38.7	36.0	47.5	35.0	39.6	Level (L)	.2196	3.9
	Mean[e]	37.3	36.2	39.2	47.2	45.0	---	T x L	.1289	5.5
Mn	10	3.32	3.35	3.26	3.15	3.67	3.35	Time (T)	.0002	.12
	20	3.97	4.00	4.02	4.19	4.41	4.12	Level (L)	.5085	.19
	Mean	3.64	3.68	3.64	3.67	4.04	---	T x L	.9450	.26
Zn	10	105	97	95	96	90	97	Time (T)	.2750	3
	20	100	104	93	108	105	102	Level (L)	.7471	5
	Mean	103	100	94	102	98	---	T x L	.6271	7
Fe	10	663	169	198	176	179	277	Time (T)	.1353	171
	20	553	664	185	839	1029	654	Level (L)	.8029	271
	Mean	608	416	192	508	604	---	T x L	.6633	383

TABLE 7-5. Continued

Mineral	Time, Days	Added Copper, mg/kg[a]						Statistics		
		0	20	40	60	80	Mean[b]	Effect	P	SE[c]
		mg/kg (dry matter basis)								
Ca	10	577[d]	613	455	539	511	539	Time (T)	.2585	41
	20	428	569	334	366	662	472	Level (L)	.1836	65
	Mean[e]	503	591	394	453	586	---	T x L	.4152	92
Mg	10	749	746	721	724	692	726	Time (T)	.0001	8
	20	843	794	831	791	808	813	Level (L)	.1685	13
	Mean	796	770	776	757	750	---	T x L	.3447	19
P	10	11570	11570	10870	11240	11200	11290	Time (T)	.0462	110
	20	11630	11360	11810	11480	11780	11610	Level (L)	.8159	170
	Mean	11598	11466	11340	11360	11490	---	T x L	.1755	240

[a]Basal diet contained 7.3 mg/kg Cu, by analysis.
[b]Each main effect mean for time represents 15 sheep.
[c]Pooled standard error.
[d]Each interaction mean represents three sheep.
[e]Each main effect mean for Cu level represents six sheep.

.10) by dietary Cu or time and averaged 41.0, 99, 466 and 505 mg/kg, respectively. Kidney Mn, Mg and P were not affected ($P > .10$) by dietary Cu, but increased with time from 3.35, 726 and 11290 mg/kg at day 10 to 4.12, 813 and 11610 mg/kg, respectively, by day 20.

Neither dietary Cu concentration nor time influenced ($P > .10$) on bone ash (Table 7-6) which averaged 66.7%. Bone Cu (Table 7-6) was not influenced ($P > .10$) by length of feeding period but was lower ($P < .05$) in sheep fed 60 mg/kg Cu than in those fed 0, 40 or 80 mg/kg Cu. Calcium concentration in bone was not influenced ($P > .10$) by dietary Cu but decreased ($P < .05$) from 34.8% at day 10 to 34.1% at day 20. Bone Mg was not affected ($P > .08$) by time, but was influenced ($P < .01$) by dietary Cu concentration. Sheep fed diets containing 20 or 40 mg/kg Cu had higher bone Mg concentrations than those fed diets containing 0, 60 or 80 mg/kg Cu. The time x level interaction for bone Mg approached significance ($P = .0541$). Phosphorus concentration in bone was not influenced ($P > .10$) by dietary Cu concentration or time and averaged 17.0%.

Discussion

The ability of sheep to maintain intake and weight at dietary Cu concentrations in excess of the maximum tolerable level suggested by the National Research Council (1980) has been previously reported (Dick, 1954; Hill and Williams, 1965; Todd, 1969; Exp. 4). This ability of sheep contrasts

TABLE 7-6. EFFECT OF TIME AND DIETARY COPPER LEVEL ON BONE ASH PERCENTAGE AND MINERAL COMPOSITION OF BONE IN SHEEP--EXPERIMENT 5

Bone Composition	Time, Days	Added Copper, mg/kg[a]						Statistics		
		0	20	40	60	80	Mean[b]	Effect	P	SE[c]
Ash, %	10	66.7[d]	66.7	66.9	67.1	66.7	66.8	Time (T)	.4919	.2
	20	66.1	66.9	66.5	66.8	66.9	66.7	Level (L)	.6383	.3
	Mean[e]	66.4	66.8	66.7	66.9	66.8	---	T x L	.7959	.4
Cu, mg/kg	10	3.43[f]	3.09	3.84	1.51	3.35	3.04	Time (T)	.3011	.23
	20	3.44	1.81	2.56	2.39	3.28	2.70	Level (L)	.0402	.37
	Mean	3.44	2.45	3.20	1.95	3.32	---	T x L	.2147	.52
Ca, %	10	35.1[f]	34.5	34.8	35.3	34.7	34.8	Time (T)	.0228	.2
	20	33.9	34.7	35.1	32.9	33.7	34.1	Level (L)	.4956	.4
	Mean	34.5	34.6	34.9	34.1	34.2	---	T x L	.0810	.5

TABLE 7-6. Continued

Bone Composition	Time, Days	Added Copper, mg/kg[a]						Statistics		
		0	20	40	60	80	Mean[b]	Effect	P	SE[c]
Mg, %	10	.647[f]	.667	.652	.626	.606	.640	Time (T)	.0861	.006
	20	.587	.632	.664	.641	.595	.624	Level (L)	.0026	.010
	Mean[e]	.617	.650	.658	.634	.601	---	T x L	.0541	.014
P, %	10	16.9[f]	16.9	16.9	17.0	17.3	17.0	Time (T)	.8942	.2
	20	16.6	17.4	17.3	17.0	16.8	17.0	Level (L)	.8273	.2
	Mean	16.8	17.1	17.1	17.0	17.0	---	T x L	.5411	.4

[a]Basal diet contained 7.3 mg/kg Cu, by analysis.
[b]Each main effect mean for time represents 15 sheep.
[c]Pooled standard error.
[d]Each interaction mean represents three sheep and is expressed as a percent of dry, fat-free bone.
[e]Each main effect mean for Cu level represents six sheep.
[f]Each interaction mean represents three sheep and is expressed on an ash weight basis.

with what is observed in most species (Owen, 1981) and may in part contribute to the greater susceptibility to Cu toxicosis exhibited by this species.

The failure of dietary Cu to influence hematocrit and hemoglobin in the present study may be attributed partially to an increase in plasma Cu concentrations observed in sheep given intravenous injections of ATM (Gooneratne et al., 1981b; Kumaratilake and Howell, 1987). Elevated plasma Cu concentrations during depletion and plasma Cu concentrations produced by dietary Cu supplementation would both ensure an adequate supply of Cu for iron mobilization and erythropoiesis. The lack of sensitivity to dietary Cu exhibited by serum in the present study is consistent with previous reports (Barden and Robertson, 1962; Todd and Thompson, 1963; Thompson and Todd, 1970; Ishmael et al., 1972) which indicated that serum Cu concentrations in sheep remain normal even if the animals were in a state of chronic Cu toxicosis and did not increase until the hemolytic crisis was imminent.

A comparison of liver Cu concentrations of Cu depleted sheep fed zero added Cu diets to those of non-depleted sheep indicated that the depletion technique was effective. Liver Cu concentrations of depleted sheep (zero added Cu diet) averaged 245 ± 18 mg/kg Cu (n = 6) while that of nondepleted sheep with the same genetic background and previous nutrition experience averaged 511 ± 120 mg/kg Cu (n = 6). These concentrations indicated that depletion with ATM for 3 weeks

decreased liver Cu by approximately 52%. Gooneratne et al. (1981a) and Kumaratilake and Howell (1987) reported liver Cu reductions of 58 and 63% in Cu loaded sheep given similar amounts of ATM for 11 weeks.

The precise mechanism by which thiomolybdate reduced liver Cu is not known. However, the increased plasma Cu levels observed in sheep given intravenous injections of ATM but fed no added dietary Cu (Gooneratne et al., 1981b; Kumaratilake and Howell, 1987) suggest that thiomolybdate causes liver Cu to be released into plasma. Most of the increased plasma Cu was found to be insoluble in trichloroacetic acid (TCA) and since this form of Cu has been shown to be unavailable for tissue uptake (Smith and Wright, 1975a, b) it would represent a loss of Cu from the liver unless there was some mechanism for its recovery. Reports of increased urinary excretion of Cu in animals with TCA insoluble Cu (Gooneratne et al., 1981b) suggest that this Cu was not recovered. Increased urinary excretion of Cu by sheep fed molybdenum and inorganic sulfate has been reported by Marcilese et al. (1970) and although plasma was not examined for the presence of TCA insoluble Cu, it is quite possible that increased urinary excretion of Cu resulted from the formation of TCA insoluble Cu since Smith and Wright (1975a, b) reported finding TCA insoluble Cu in the plasma of sheep fed added dietary molybdenum. In addition to increased urinary excretion of Cu, intravenous injections of ATM have also been shown to increase fecal excretion

(Gooneratne et al., 1981a, b). Gooneratne et al. (1985) presented evidence to show that this resulted from enhanced biliary excretion and not from endogenous secretions or excretion of unabsorbed dietary Cu. They hypothesized that a compound similar to that found in plasma TCA insoluble Cu is formed in the liver and then excreted in bile. Although it is apparent that increased urinary and biliary excretion of Cu contributes to the reduction in liver Cu stores, Gooneratne et al. (1985) contend that quantitatively these two systems cannot account for all of the reduction observed.

Of tissues evaluated, liver proved to be the most sensitive to dietary Cu with liver Cu accumulating linearly in sheep fed diets for 10 days and quadratically in sheep fed diets for 20 days. The greater sensitivity of liver to dietary Cu is compatible with previous reports (Dick, 1954; Hill and Williams, 1965; Hemingway and MacPherson, 1967; Exp. 1, 2, 3 and 4) and is in concurrence with the observation of Underwood (1977) that liver is the main storage organ for Cu in the body. The linear accumulation of Cu in the liver of sheep fed diets for 10 days suggested that the 10 day bioassay would be the assay of choice since a linear relationship allows the use of a slope ratio technique to determine relative bioavailability and precludes the use of a non-linear model with its inherent complexities.

Todd (1969) contends that with the exception of liver, Cu does not accumulate in the tissues of sheep unless

animals have undergone a hemolytic crisis and then only in kidney and to a lesser extent spleen. Gopinath et al. (1974) and Gooneratne and Howell (1985) found that hemolysis was not a prerequisite for Cu accumulation in kidney. However, a consistent finding in these and other studies (Barden and Robertson, 1962; Ishmael et al., 1971, 1972; Theil and Calvert, 1978) citing elevated kidney and spleen Cu concentrations was the extremely high levels of Cu observed in livers of these animals. In all cases liver Cu concentrations were in excess of 2000 mg/kg on a dry matter basis (DM), a concentration considered symptomatic of Cu toxicosis. These data suggested that Cu did not accumulate in the kidney and spleen of sheep unless the animals were in a state of chronic Cu toxicosis and explained the lack of sensitivity to dietary Cu exhibited by kidney and spleen in the present study, where liver Cu concentrations averaged only 433 mg/kg (DM). A similar lack of sensitivity to dietary Cu was exhibited by kidney and spleen in a previous experiment (Exp. 4) with sheep in which liver Cu averaged 503 mg/kg (DM).

Bones were thought to be particularly responsive to changes in dietary Cu concentrations (Underwood, 1977; Georgievskii, 1982). However, results of this and previous studies suggest that this response varies with species and Cu status. At Cu intakes below requirement, Cu supplementation resulted in increased bone Cu concentrations in rats (Lindow et al., 1929), chicks (Rucker et al., 1969), cattle

and sheep (Hennig et al., 1974). However, at dietary Cu levels in excess of requirements, Cu supplementation had no effect on bone Cu in pigs (Allen and Harding, 1962; Hedges and Kornegay, 1973) or chicks (Exp. 1 and 2), but did increase bone Cu in rats (Lal and Sourkes, 1971). In the present study, the response to dietary Cu varied with Cu concentration; bone Cu concentrations in sheep fed 60 mg/kg Cu were similar to those fed 20 mg/kg Cu but lower than that of sheep fed 0, 40 or 80 mg/kg Cu. The response of bone Cu to increasing age also appeared to vary with species. In the Cu loaded rat, bone Cu has been reported to decline with age except in situations of prolonged loading (Lal and Sourkes, 1971). A similar decline was not observed in the present study or in previous studies with sheep (Exp. 4) or chicks (Exp. 2).

Other than Cu, the only tissue mineral to be affected by dietary Cu was bone Mg. Bone Mg concentrations in sheep fed 80 mg/kg Cu were similar to sheep fed no supplemental Cu but lower than that of sheep fed 20, 40 or 60 mg/kg Cu. Dietary Cu had no effect on bone Mg in previous experiments with day-old chicks (Exp. 1, 2 or 3) and sheep (Exp. 4). Tissue minerals affected by length of feeding period included serum Zn, liver Mn, Zn, Mg, P and Ca, kidney Mn, Mg and P and bone Ca. However, unlike a previous experiment (Exp. 4) in which the response to length of feeding period was consistent across all tissues, the response was consistent only within tissues in the present study.

Mineral concentrations in serum and kidney increased with time, while that of liver and bone decreased with time. In both studies liver Mg and P and bone Ca all decreased with time.

Results of the present study suggest that liver Cu accumulation may be a useful bioassay criterion for determining Cu bioavailability and that a 10 day bioassay would be acceptable. However, the design of the study was such that it is not known whether the depletion technique was useful in helping to promote liver Cu storage during the repletion phase.

Summary

Thirty mature crossbred wethers averaging 62 kg initially were used to study the effect of time and dietary Cu concentration on tissue Cu accumulation and tissue mineral composition of sheep depleted of Cu for 3 weeks. During the depletion phase sheep were housed in two large covered floor pens and group-fed a corn-soybean meal-cottonseed hulls basal diet containing 6.4 mg/kg Cu (as fed basis). Animals were depleted of Cu by intravenous injection of 50 mg ammonium tetrathiomolybdate (ATM) twice weekly for 3 weeks. Following the depletion phase animals were placed in individual raised, slotted floor pens and fed the basal diet for 5 days in order to allow animals time to clear their systems of ATM and to adapt to the individual pens. At the end of the adjustment period, animals were

assigned randomly to a 5 x 2 factorial arrangement of treatments which included the basal diet supplemented with 0, 20, 40, 60 or 80 mg/kg Cu from reagent grade cupric acetate [$Cu(C_2H_3O_2)_2 \cdot H_2O$] fed for 10 or 20 days. Feed intake was restricted to 1200 g daily and tap water was available ad libitum. At the end of each time period, blood samples were obtained by jugular vein puncture, animals euthanized and liver, spleen, both kidneys and right metacarpus excised and frozen for subsequent mineral analysis. Copper depletion resulted in a 52% reduction in liver Cu concentrations. Hemoglobin and hematocrit were not affected ($P > .10$) by dietary Cu or time and averaged 12.4 g/dl and 33.4%, respectively. Copper concentrations in liver increased ($P < .01$) with increasing dietary Cu in both feeding periods. Regression of liver Cu on dietary Cu by time indicated that the increase in Cu at day 10 was linear ($P < .01$, $R = .57$) while that at day 20 was quadratic ($P < .01$, $R = .69$). Bone Cu was not influenced ($P > .10$) by time but was lower ($P < .05$) in sheep fed 60 mg/kg Cu compared to those fed 0, 40 or 80 mg/kg Cu. Copper concentrations in spleen and kidney were not affected ($P > .10$) by dietary Cu or time averaging 6.89 and 41.0 mg/kg, respectively. Bone Mg was the only tissue mineral other than Cu to be affected by dietary Cu, with sheep fed diets containing 20 or 40 mg/kg Cu having higher bone Mg concentrations than those fed diets containing 0, 60 or 80 mg/kg Cu. Tissue minerals affected by length of feeding period

included serum Zn, liver Mn, Zn, Mg, P and Ca, kidney Mn, Mg and P and Bone Ca. Data indicated that liver Cu accumulation may be a useful bioassay criterion for determining Cu bioavailability and that a 10 day bioassay was optimum.

CHAPTER VIII
BIOLOGICAL AVAILABILITY OF COPPER FROM INORGANIC SOURCES FED AT HIGH DIETARY LEVELS TO SHEEP DEPLETED OF COPPER FOR THREE WEEKS--EXPERIMENT 6

Introduction

Evidence for an essential physiological role for Cu in animals was first provided by Hart et al. (1928) when they demonstrated that both Cu and Fe were required for hemoglobin formation in anemic rats. Three years later Cu deficiency in grazing cattle was demonstrated in Florida (Neal et al., 1931) and by 1939 "neonatal ataxia" (swayback) of lambs (Bennetts and Chapman, 1937) and "falling disease" of cattle (Bennetts and Hall, 1939) were both recognized as Cu deficiency syndromes that could be prevented by Cu supplementation. The requirement for Cu is relatively small and, as such, economic considerations concerning choice of a feed grade supplement may not be critical. However, the chance of a Cu deficiency occurring with resultant economic losses requires consideration of the biological availability of these sources (McNaughton et al., 1974).

Attempts to determine Cu availability from inorganic sources, using traditional methods have met with limited success (Miller, 1983). Bioassays are complicated by Cu absorption and retention values that are low relative to

that of other elements and by interactions between Cu and other dietary consitiuents.

Results of a previous experiment (Exp. 5) indicated that Cu accumulation in the liver of sheep depleted of Cu for 3 weeks may be a useful bioassay criterion for determining Cu availability from inorganic sources.

This experiment was conducted to determine if this bioassay technique could be used successfully to determine Cu availability from inorganic feed grade sources of Cu.

Materials and Methods

Thirty-two mature crossbred wethers averaging 54 kg initially were used to determine the biological availability of Cu from inorganic sources fed at high dietary levels to sheep depleted of Cu for 3 weeks. Initially, all sheep were housed in two large covered floor pens and group fed a corn-soybean meal-cottonseed hulls basal diet at an amount selected to ensure an average daily intake of not more than 1100 g per animal. The basal diet (Table 8-1) contained 8.7 mg/kg Cu (dry basis) by analysis and was formulated to meet the nutrient requirements of sheep (NRC, 1985). Animals were depleted of Cu by intravenous injections of 50 mg ammonium tetrathiomolybdate (ATM) twice weekly for 3 weeks.

Ammonium tetrathiomolybdate was prepared by bubbling hydrogen sulfide gas through a solution of ammonium molybdate until crystals of ATM were formed. The ammonium molybdate solution was prepared by dissolving ammonium

TABLE 8-1. COMPOSITION OF BASAL DIET--EXPERIMENT 6

Item	%
Ingredient composition[a]	
Corn, ground yellow	58.5
Soybean meal	12.0
Cotton, seed hulls	21.0
Alfalfa, dehy meal	3.0
Corn, oil	3.0
Corn, starch	.95
Limestone, ground	.55
Salt	.50
Salt, trace mineralized[b]	.50
Vitamin, A, D[c]	+
TOTAL	100.00
Chemical composition[d]	
Dry matter, %	88.1
Crude protein, %	12.3
Crude fiber, %	11.7
Ca, %	.45
P, %	.29
Mg, %	.16
Cu, mg/kg[e]	8.65
Mn, mg/kg	16.3
Zn, mg/kg	87
Fe, mg/kg	241

[a] As-fed basis. Santoquin added at .0125% of total diet.

[b] Minerals supplied per kilogram of diet: Copper, 5 mg; iron, 100 mg; zinc, 50 mg; cobalt, 2.5 mg; iodine, 2.5 mg.

[c] Vitamin A, 2200 USP units/kg; vitamin D, 440 USP units/kg.

[d] Dry matter basis. Crude protein and fiber calculated, dry matter and minerals determined by analysis.

[e] Copper as $Cu[C_2H_3O_2]_2 \cdot H_2O$ added on an air dry basis at expense of corn starch.

molybdate (8.6 g) in ammonium hydroxide (106 ml) and water (24 ml). The orange crystals of ATM formed were first washed with cold distilled water followed by cold acetone then dried under a vacuum and stored at -4°C. Injectable solutions of ATM were prepared weekly by dissolving crystals in sterile saline (.9%) to produce a 25 mg/ml solution.

Following the depletion phase, animals were fed the basal diet for five more days in order to allow them time to clear their system of ATM. Liver samples were then collected by biopsy using a laparotomy technique. With this technique, following shaving and prepping of the left flank area, animals were tranquilized with xylazine (.044 mg/kg BW) and line blocked with lidocaine. A laparotomy incision was then made and samples collected by manually crushing and tearing off approximately 5 g (wet weight) of liver tissue. The procedure was then completed by a two-layer closure of the incision.

Following biopsy, animals were returned to floor pens and fed the basal diet for 12 days to allow recovery from surgery trauma. Animals were then placed in individual raised, slotted floor pens and fed the basal diet (1100 g/d) for a further 7 days to allow animals time to adjust to pens. At the end of the adjustment period, animals were assigned randomly to one of eight experimental diets for a period of 10 days. Dietary treatments included the basal diet supplemented with either 0, 20, 40, 60 or 80 mg/kg Cu from reagent grade cupric acetate (31.8% Cu) or 60 mg/kg Cu

from feed grade oxide (74.1% Cu), feed grade sulfate (25.1% Cu) or feed grade carbonate (54.6% Cu). Final dietary Cu concentrations were confirmed by chemical analysis. Feed intake was restricted to 1100 g daily and tap water was available ad libitum. Relative solubilities (Watson et al., 1970), magnetic susceptibility (Watson et al., 1971), chemical and physical characteristics of Cu sources were determined and x-ray diffraction patterns were interpreted (Table 8-2).

At the conclusion of the 10 day feeding period, blood samples were obtained by jugular vein puncture, animals were stunned, killed by exsanguination and liver, spleen, both kidneys and right metacarpus excised and frozen for subsequent mineral analysis. Copper, Mn, Zn, Fe, Ca and Mg concentrations in feed, tissues and Cu sources were determined by flame atomic absorption spectrophotometry on a Perkin-Elmer Model 5000 with an AS-50 Autosampler (Perkin-Elmer Corporation, 1982). Standards were matched for macroelement and acid concentrations as needed and standard reference material from the National Bureau of Standards was included with samples. Phosphorus was determined by a modified colorimetric procedure (Harris and Popat, 1954). Hemoglobin was determined by a cyanomethemoglobin method (Unopette Test 5857/5858; Becton-Dilkinson, Rutherford, NJ) and hematocrit by a micro-hematocrit method (Blu-tip micro-hematocrit capillary tubes, Sherwood medical industries, St. Louis, MO).

TABLE 8-2. CHEMICAL AND PHYSICAL CHARACTERISTICS OF COPPER SOURCES--EXPERIMENT 6

	Copper Source			
	Acetate, Reagent Grade	Oxide, Feed Grade	Sulfate, Feed Grade	Carbonate, Feed Grade
Chemical Constituents, Dry Matter Basis				
Cu, %	32.1	74.1	25.1	54.6
Ca, mg/kg	---[a]	289	---	200
P, mg/kg	---	---	---	---
Mg, mg/kg	---	2140	---	212
Zn, mg/kg	---	688	7	565
Fe, mg/kg	---	5430	76	3020
Mn, mg/kg	---	45.8	---	36.3
Particle Size, %[b]				
+30	4.0	---	.6	---
-30 + 100	78.9	.3	94.2	.3
-100	17.1	99.7	5.2	99.7

TABLE 8-2. Continued

	Copper Source			
	Acetate, Reagent Grade	Oxide, Feed Grade	Sulfate, Feed Grade	Carbonate, Feed Grade
Physical Appearance	Dark blue, fine crystals	Black, fine powder	Light blue, crystals	Light green, fine powder
Relative Solubility, %[c]				
Water	95.2	---	98.9	---
Neutral Ammonium Citrate	98.8	4.9	98.8	98.7
HCl, .4%	99.9	59.1	95.5	97.7
Citric Acid, 2%	100	8.3	100	97.9
Magnetic Susceptibility, %	---	25.2	---	5.0
Interpretation of X-Ray Patterns[d]	$Cu[C_2H_3O_2]_2 \cdot H_2O$	CuO plus minor Cu_2O	$CuSO_4 \cdot 5H_2O$	$Cu_2(OH)_2CO_3$

[a] None detected.
[b] Retained by a No.30 sieve (U.S. Bureau of Standards).
[c] Solubility in solvents at 37°C for 1 hour with constant stirring expressed as % of total mineral content.
[d] Courtesy of International Minerals and Chemical Corporation, Fertilizer Group, Noralyn, Bartow, Florida.

Data were analyzed by one-way analysis of variance using the general linear models procedure of SAS (1982).

Results and Discussion

Chemical and physical characteristics of mineral sources were determined and are presented in Table 8-2. Analysis of mineral sources indicated Cu concentrations of 32.1, 74.1, 25.1 and 54.6% for the acetate, oxide, sulfate and carbonate sources, respectively. No mineral contaminants were detected in the reagent grade acetate, but a comparison of the feed grade sources indicated that the oxide and carbonate sources contained a number of other elements. The relatively low magnetic susceptibility observed in the oxide and carbonate sources may be related to the Fe content of these sources. Interpretation of x-ray diffraction patterns indicated a high degree of purity for the acetate, sulfate and carbonate sources, while the oxide was primarily cupric oxide (CuO) with a minor portion of cuprous oxide (Cu_2O). A low proportion of cuprous oxide is desirable since this form is less available than the cupric form.

In addition to in vivo techniques, several in vitro techniques have been developed in an attempt to predict bioavailability. Solubility tests have proved by far to be the most effective. Relative solubility tests conducted in the present study (Table 8-2) indicated that the acetate and sulfate sources were almost completely soluble in water,

while the oxide and carbonate sources were insoluble. The acetate, sulfate and carbonate sources had essentially the same solubility (> 95%) in neutral ammonium citrate, dilute (.4%) hydrochloril acid and dilute (2%) citric acid. The oxide was the least soluble of the sources tested, being insoluble in water and with solubilities of 4.9, 59.1 and 8.3% in neutral ammonium citrate, hydrochloric acid and citric acid, respectively. Solubility did not appear to be related to particle size. Based on the results of solubility tests, Cu from reagent grade acetate, feed grade sulfate and feed grade carbonate appeared to be equally available, whereas that from feed grade oxide was less available.

Hemoglobin and hematocrit (Table 8-3) were not affected ($P > .10$) by dietary treatment and averaged 13.7 g/dl and 37.3%, respectively. However, when sources were compared at equivalent dietary Cu concentrations (60 mg/kg), sheep fed oxide and sulfate sources had numerically less hemoglobin (8.5 and 9.9%, respectively) and lower hematocrits (6.8 and 8.3%, respectively) than those fed acetate or carbonate sources. This suggested that acetate and carbonate sources may have been more efficient in promoting hemoglobin regeneration and erythropoiesis in response to blood loss that occurred during biopsy surgery.

Serum concentrations of Cu, Zn, Fe, Ca, Mg and P (Table 8-4) were not affected ($P > .10$) by dietary tratment and averaged 110 µg/dl, 84 µg/dl, 216 µg/dl, 9.6 mg/dl, 2.21

TABLE 8-3. EFFECT OF SOURCE AND LEVEL OF DIETARY COPPER ON HEMOGLOBIN AND HEMATOCRIT IN SHEEP--EXPERIMENT 6

Added Cu, mg/kg[a]	Copper Source	Hemoglobin g/dl	Hematocrit %
0	---	13.6[b]	38.7
20	Acetate[c]	13.5	38.2
40	Acetate	13.2	37.4
60	Acetate	14.1	38.4
80	Acetate	13.7	36.7
60	Oxide[d]	12.9	35.8
60	Sulfate	12.7	35.2
60	Carbonate	14.0	38.3
SE[e]		0.8	1.5

[a]Basal diet contained 8.7 mg/kg Cu, by analysis.

[b]Each mean represents four sheep.

[c]Reagent grade.

[d]Feed grade.

[e]Pooled standard error.

TABLE 8-4. EFFECT OF SOURCE AND LEVEL OF DIETARY COPPER ON THE MINERAL COMPOSITION OF SERUM IN SHEEP--EXPERIMENT 6

Added Cu mg/kg[a]	Copper Source	Mineral Concentration					
		μg/dl			mg/dl		
		Cu	Zn	Fe	Ca	Mg	P
0	---	94[b]	86	185	9.6	2.37	7.52
20	Acetate[c]	113	80	250	9.7	2.07	6.57
40	Acetate	128	87	177	9.5	2.32	8.04
60	Acetate	105	90	249	9.6	2.25	7.84
80	Acetate	109	72	245	9.6	2.22	7.10
60	Oxide[d]	101	78	184	9.5	2.16	8.14
60	Sulfate[d]	109	86	251	9.7	2.14	7.24
60	Carbonate[d]	122	96	187	9.9	2.19	7.30
SE[e]		8	10	28	.2	.10	.54

[a]Basal diet contained 8.7 mg/kg Cu, by analysis.
[b]Each mean represents four sheep.
[c]Reagent grade.
[d]Feed grade.
[e]Pooled standard error.

mg/dl and 7.47 mg/dl, respectively. The lack of sensitivity to dietary Cu exhibited by serum in this and previous studies with sheep (Exp. 4 and 5) is consistent with reports (Barden and Robertson, 1962; Todd and Thompson, 1963; Thompson and Todd, 1970; Ishmael et al., 1972) which indicated that serum Cu concentrations in sheep remain normal even when animals were in a state of chronic Cu toxicosis and did not increase until the hemolytic crisis was imminent. Increased dietary Cu concentrations also failed to affect serum concentrations of Zn, Mg and P in previous experiments with sheep (Exp. 4 and 5) and chicks (Exp. 1, 2 and 3).

Final liver Cu concentrations (Table 8-5) were not affected ($P > .10$) by dietary treatment and averaged 542 mg/kg. The ratios of final liver Cu concentrations to initial liver Cu concentrations (Table 8-6) were also not affected ($P > .10$) by dietary treatment. However, ratios increased numerically with increasing dietary Cu in sheep fed acetate and at equivalent dietary Cu concentrations (60 mg/kg) the carbonate source had the widest ratio numerically, followed by acetate, sulfate and oxide, respectively. These results contrast with a previous experiment (Exp. 5) in which liver Cu concentrations were found to increase linearly with increasing dietary Cu in sheep fed similar levels of Cu for 10 days. The failure of added dietary Cu to increase liver Cu concentrations in the present study may have occurred as a result of surgery trauma since animals

TABLE 8-5. EFFECT OF SOURCE AND LEVEL OF DIETARY COPPER ON THE MINERAL COMPOSITION OF LIVER IN SHEEP--EXPERIMENT 6

Added Cu mg/kg[a]	Copper Source	Mineral Concentration (mg/kg, dry matter basis)[b]						
		Cu	Mn	Zn	Fe	Ca	Mg	P
0	---	490	10.93	118	217	149	565	11180
20	Acetate[c]	624	9.36	125	193	157	590	11530
40	Acetate	605	9.65	124	149	153	586	11420
60	Acetate	567	9.81	120	221	161	579	11340
80	Acetate	598	10.65	127	200	175	636	12210
60	Oxide[d]	480	9.12	126	131	150	580	11100
60	Sulfate[d]	513	10.06	123	216	161	597	11750
60	Carbonate[d]	459	9.26	135	260	163	609	11860
SE[e]		80	.61	10	48	8	24	270

[a]Basal diet contained 8.7 mg/kg Cu, by analysis.
[b]Each mean represents four sheep.
[c]Reagent grade.
[d]Feed grade.
[e]Pooled standard error.

TABLE 8-6. EFFECT OF SOURCE AND LEVEL OF DIETARY COPPER ON LIVER COPPER ACCUMULATION IN SHEEP--EXPERIMENT 6

Added Cu, mg/kg[a]	Copper Source	Initial Cu, mg/kg	Final Cu, mg/kg	Ratio F/I
0	---	431[b]	490	1.10
20	Acetate[c]	532	624	1.17
40	Acetate	475	605	1.28
60	Acetate	458	567	1.28
80	Acetate	413	598	1.66
60	Oxide[d]	444	480	1.13
60	Sulfate[d]	449	513	1.21
60	Carbonate[d]	369	459	1.52
SE[e]		81	80	.20

[a]Basal diet contained 8.7 mg/kg Cu, by analysis.

[b]Each mean represents four sheep.

[c]Reagent grade.

[d]Feed grade.

[e]Pooled standard error.

were put on experiment within 19 days of surgery. Liver concentrations of Zn, Fe, Ca, Mg and P (Table 8-5) were not affected ($P > .10$) by dietary treatment and averaged 125, 198, 159, 593 and 11550 mg/kg, respectively. With the exception of Mg in Exp. 4, these results are similar to those of Exp. 4 and 5.

Copper concentrations in spleen (Table 8-7) and kidney (Table 8-8) were not affected ($P > .10$) by dietary treatment and averaged 10.21 and 38.5 mg/kg, respectively. These results, while being similar to those obtained in Exp. 4 and 5, are at variance with previous reports (Barden and Robertson, 1962; Ishmael et al., 1971, 1972; Theil and Calvert, 1978) citing elevated kidney and spleen Cu concentrations in sheep fed high dietary levels of Cu. However, Todd (1969) may have supplied an explanation for this contradiction when he contended that with the exception of liver, Cu does not accumulate in tissues of sheep unless animals have undergone a hemolytic crisis and then only in kidney and to a lesser extent spleen. This contention was subsequently modified by Gopinath et al. (1974) and Gooneratne and Howell (1985) who showed that sheep in a state of chronic Cu toxicosis can accumulate Cu in their kidneys without a hemolytic crisis occurring. These data suggest that Cu does not accumulate in tissues other than liver unless animals are in a state of chronic Cu toxicosis. A comparison of liver Cu concentrations of sheep used in the present study and in Exp. 4 and 5 to those of sheep used in

TABLE 8-7. EFFECT OF SOURCE AND LEVEL OF DIETARY COPPER ON THE MINERAL COMPOSITION OF SPLEEN IN SHEEP--EXPERIMENT 6

Added Cu mg/kg[a]	Copper Source	Mineral Concentration (mg/kg, matter dry basis)						
		Cu	Mn	Zn	Fe	Ca	Mg	P
0	---	9.14[b]	1.50	101	1103	200	888	13320
20	Acetate[c]	11.39	1.54	101	2058	198	869	12670
40	Acetate	10.82	1.41	107	976	213	856	13070
60	Acetate	11.14	1.47	112	665	216	850	13270
80	Acetate	10.26	1.53	99	2097	212	866	13910
60	Oxide[d]	10.05	1.33	106	546	203	847	13220
60	Sulfate[d]	9.98	1.36	102	1098	217	838	13160
60	Carbonate[d]	8.92	1.45	105	796	215	825	13370
SE[e]		.89	.09	4	594	11	17	320

[a]Basal diet contained 8.7 mg/kg Cu, by analysis.
[b]Each mean represents four sheep.
[c]Reagent grade.
[d]Feed grade.
[e]Pooled standard error.

TABLE 8-8. EFFECT OF SOURCE AND LEVEL OF DIETARY COPPER ON THE MINERAL COMPOSITION OF KIDNEY IN SHEEP--EXPERIMENT 6

Added Cu, mg/kg[a]	Copper Source	Mineral Concentration (mg/kg, dry matter basis)						
		Cu	Mn	Zn	Fe	Ca	Mg	P
0	---	39.0[b]	4.89	99	349	558	759	11330
20	Acetate[c]	39.6	4.44	108	188	447	770	11490
40	Acetate	36.0	3.95	104	624	543	793	11440
60	Acetate	43.4	4.72	104	674	496	780	11240
80	Acetate	39.2	4.55	107	226	546	782	11370
60	Oxide[d]	40.2	5.12	125	162	731	812	11630
60	Sulfate[d]	33.9	4.39	101	591	750	763	11220
60	Carbonate[d]	36.5	4.22	120	263	483	770	11340
SE[e]		3.8	.27	10	285	106	19	260

[a]Basal diet contained 8.7 mg/kg Cu, by analysis.
[b]Each mean represents four sheep.
[c]Reagent grade.
[d]Feed grade.
[e]Pooled standard error.

studies citing elevated kidney and spleen Cu concentrations appear to confirm this contention. In the present study and in Exp. 4 and 5, liver Cu concentrations averaged 542, 503 and 433 mg/kg (DM), respectively, levels considered to be within normal ranges; whereas in studies citing elevated kidney and spleen Cu concentrations, liver Cu concentrations were in excess of 2000 mg/kg (DM), a concentration symptomatic of Cu toxicosis. No other mineral constituents of spleen (Table 8-7) and kidney (Table 8-8) were affected ($P > .10$) by dietary treatment in the present study. Similar results were obtained in previous experiments with sheep (Exp. 4 and 5).

Bone ash percentage (Table 8-9) was not affected ($P > .10$) by dietary treatment and averaged 66.9%. Dietary Cu also had no effect on bone ash percentage in previous experiments with sheep (Exp. 4 and 5) or chicks (Exp. 1 and 3). However, in a previous experiment (Exp. 3) chicks fed cupric acetate had lower percentage bone ash than did those fed oxide or carbonate sources. This reduction in bone ash, however, appeared to be due to a Cu mediated reduction in feed intake and growth and not to Cu per se.

Bone Cu (Table 8-9) was not affected ($P > .10$) by dietary treatment and averaged 2.02 mg/kg. Bone was thought to be particularly responsive to changes in dietary Cu concentrations (Underwood, 1977; Georgievskii, 1982). However, results of this and previous studies suggest that this response varies with species and Cu status. At Cu

TABLE 8-9. EFFECT OF SOURCE AND LEVEL OF DIETARY COPPER ON BONE ASH PERCENTAGE AND MINERAL COMPOSITION OF BONE IN SHEEP--EXPERIMENT 6

Added Cu, mg/kg[a]	Copper Source	Bone Ash, % Dry, Fat-Free Bone	Mineral Composition			
			Cu, mg/kg	Mg, %	Ca, %	P, %
0	---	66.9[b]	2.20[c]	.593	33.8	17.0
20	Acetate[d]	66.3	1.94	.572	34.3	17.0
40	Acetate	67.3	1.96	.611	33.9	16.9
60	Acetate	67.3	2.07	.624	34.5	17.4
80	Acetate	66.8	1.82	.614	34.1	17.1
60	Oxide[e]	66.4	2.02	.602	33.9	17.0
60	Sulfate[e]	66.4	2.01	.615	34.4	17.0
60	Carbonate[e]	67.5	2.16	.585	34.7	17.1
SE[f]		.3	.15	.015	.3	.2

[a]Basal diet contained 8.7 mg/kg Cu, by analysis.
[b]Each mean represents four sheep.
[c]Each mean represents four sheep and is expressed on an ash weight basis.
[d]Reagent grade.
[e]Feed grade.
[f]Pooled standard error.

intakes below requirement, Cu supplementation resulted in increased bone Cu concentrations in rats (Lindow et al., 1929), chicks (Rucker et al., 1969), cattle and sheep (Hennig et al., 1974). However, at dietary Cu levels in excess of requirements, Cu supplementation had no effect on bone Cu in pigs (Allen and Harding, 1962; Hedges and Kornegay, 1973) or chicks (Exp. 1 and 2), but did increase bone Cu in rats (Lal and Sourkes, 1971). Bone concentrations of Ca, Mg and P (Table 8-9) were also not affected ($P > .10$) by dietary treatment averaging 34.2, .602 and 17.1%, respectively. Dietary Cu also failed to affect bone concentrations of Ca and P in previous experiments with sheep (Exp. 4 and 5).

Results of the present study indicate that at a dietary Cu concentration of 60 mg/kg, tissue Cu accumulation in sheep fed diets for 10 days was not a sensitive enough bioassay criterion to be able to determine Cu availability from inorganic sources. However, since similar dietary concentrations of Cu fed for 10 days resulted in a linear increase in liver Cu in a previous experiment with sheep, it is suggested that surgery trauma may have confounded the results of the present study.

Summary

Thirty-two mature crossbred wethers averaging 54 kg initially were used to determine biological availability of Cu from inorganic sources fed at high dietary levels to

sheep depleted of Cu for 3 weeks. During the depletion phase, sheep were housed in two large covered floor pens and group-fed a corn-soybean meal-cottonseed hulls basal diet containing 7.62 mg/kg Cu (as fed basis). Animals were depleted of Cu by intravenous injection of 50 mg ammonium tetrathiomolybdate (ATM) twice weekly for 3 weeks. Following the depletion phase, animals were fed the basal diet for five more days in order to allow time to clear their circulatory systems of ATM. Animals were then biopsied (liver) using a laparotomy technique. Following biopsies, animals were allowed 12 days to recover from surgery then placed into individual raised, slotted floor pens and fed the basal diet for a further 7 day adjustment. At the end of this adjustment period animals were assigned randomly to one of eight dietary treatments (four sheep/ treatment) for a period of 10 days. Dietary treatments included the basal diet supplemented with either 0, 20, 40, 60 or 80 mg/kg Cu from reagent grade cupric acetate or 60 mg/kg Cu from feed grade oxide, sulfate or carbonate. Feed intake was restricted to 1100 g daily and tap water was available ad libitum. At the conclusion of the 10 day feeding period, blood samples were obtained by jugular vein puncture, animals were stunned, killed by exsanguination and liver, spleen, both kidneys and right metacarpus excised and frozen for subsequent mineral analysis. Analysis of the data indicated that dietary treatment had no influence ($P > .10$) on any of the variables measured. However, the ratio

of final Cu concentration to initial Cu concentration increased numerically with increasing dietary Cu in sheep fed acetate and at equivalent dietary Cu concentrations (60 mg/kg) the carbonate source had the widest ratio, followed by acetate, sulfate and oxide sources, respectively. Since similar dietary levels of Cu fed for 10 days resulted in a linear increase in liver Cu in a previous experiment with sheep, it is suggested that surgery trauma may have confounded results of the present study.

CHAPTER IX
GENERAL SUMMARY AND CONCLUSIONS

Six experiments were conducted to investigate the possibility of using tissue Cu accumulation during short-term, high level Cu supplementation to determine Cu availability from inorganic sources fed to chicks and sheep. In Experiment 1, chicks were fed diets supplemented with 0, 100, 200 or 300 mg/kg Cu as reagent grade cupric acetate for 22 days to determine the relationship between dietary Cu and tissue Cu and to evaluate the sensitivity of various tissues for use in a bioavailability assay. Feed intake gain and feed conversion were not affected ($P > .10$) by dietary Cu. Copper concentrations in liver and plasma increased ($P < .001$) with increasing dietary Cu, while that of kidney and bone remained unchanged ($P > .10$). Regression analysis indicated that the increase in plasma Cu concentrations was linear ($P < .001$) while that of liver was quadratic ($P < .001$) in nature. Other tissue minerals affected by dietary Cu included liver Mn and P, kidney Fe and Mn and bone Ca and P. Data indicated that liver Cu accumulation may be a useful bioassay criterion for determining Cu bioavailability from inorganic sources.

Experiment 2 was conducted to determine the effect of age on tissue Cu accumulation and tissue mineral composition

and to evaluate the sensitivity of various tissues for use in a bioavailability assay. Reagent grade cupric acetate was fed to chicks at 0, 100, 200 or 300 mg/kg for 1, 2 or 3 weeks. In contrast to Experiment 1, feed intake and gain were lower ($P < .01$) at week 3 for chicks fed 300 mg/kg Cu, suggesting a marginal Cu toxicosis. Liver proved to be the only tissue sensitive to dietary Cu with Cu concentrations increasing ($P < .001$) with increasing dietary Cu at weeks 2 and 3. Regression of log transformed liver Cu on dietary Cu by age indicated that this increase was quadratic ($P < .001$). Liver Cu was not affected ($P > .10$) by dietary Cu at week 1. Other tissue minerals affected by dietary Cu include liver Zn, Fe and Ca, kidney Mn and Mg, muscle Fe and plasma Ca. With the exception of muscle in which all minerals decreased ($P < .001$) with age, tissue mineral response to age varied with tissue and mineral within tissue. Data indicated that liver Cu accumulation may be a useful bioassay criterion for determining Cu bioavailability and that 2 weeks is the minimum time required for such an assay in chicks.

Experiment 3 was conducted to determine if the new assay could be used to measure Cu availability from inorganic sources of Cu. Inorganic sources included reagent grade cupric acetate (31.8% Cu), feed grade oxide (74.1% Cu), feed grade carbonate (54.6% Cu) and feed grade sulfate (25.1% Cu). Copper sources were fed to chicks at dietary concentrations of 0, 150, 300 or 450 mg/kg Cu for 21 days.

Feed intake, gain and feed conversion were not affected ($P > .10$) by dietary Cu concentration. However, chicks fed acetate consumed less feed ($P < .10$) and had lower gains ($P < .001$) than those fed oxide, carbonate or basal diets. Liver Cu was not affected by dietary Cu from the oxide source but increased ($P < .001$) with increasing dietary Cu from all other sources. Regression analysis of liver Cu with respect to dietary Cu indicated a significant linear relationship ($R = .88$) between log transformed liver Cu concentrations and dietary Cu. Using the slope-ratio technique with acetate set at 100%, the relative biological availability values were determined to be 98.3 and 67.7% for the sulfate and carbonate sources, respectively, with the oxide source being essentially unavailable. Alternatively, if average increase in liver Cu concentration over basal is used for comparison, then the relative biological availability values were 103.2 and 35% for the sulfate and carbonate sources, respectively, with the oxide source found to be essentially unavailable.

In Experiment 4, sheep were fed 0, 15, 30 or 45 mg/kg added Cu as reagent grade cupric acetate for 15 or 30 days to determine the relationship among time, dietary Cu and tissue Cu and to evaluate the sensitivity of various tissues for use in a bioavailability assay. Data indicated that at dietary Cu concentrations of 15 to 45 mg/kg homeostatic mechanisms in the tissues of sheep appear capable of maintaining Cu concentrations within normal ranges for periods

in excess of 30 days and as such an assay based on tissue Cu accumulation would be ineffective unless feeding periods were extended.

Experiment 5 was conducted to determine the relationship among time, dietary Cu and tissue Cu in sheep depleted of Cu for 3 weeks. Sheep were depleted of Cu by intravenous injections of 50 mg ammonium tetrathiomolybdate (ATM) twice weekly for 3 weeks, then supplemented with 0, 20, 40, 60 or 80 mg/kg Cu as reagent grade cupric acetate for 10 or 20 days. Copper depletion resulted in an estimated 52% reduction in liver Cu concentrations. Hemoglobin and hematocrit were not affected ($P > .10$) by dietary Cu or time and averaged 12.4 g/dl and 33.4%, respectively. Copper concentrations in liver increased ($P < .01$) with increasing dietary Cu in both feeding periods. Regression of liver Cu on dietary Cu by time indicated that the increase in Cu at day 10 was linear ($P < .10$, $R = .57$) while that at day 20 was quadratic ($P < .01$, $R = .69$). Bone Cu was lower ($P < .05$) in sheep fed 60 mg/kg Cu compared to those fed 0, 40 or 80 mg/kg Cu and was the only tissue other than liver in which Cu concentrations were affected by dietary Cu. Bone Mg was the only tissue mineral other than Cu to be affected by dietary Cu, with sheep fed diets containing 20 or 40 mg/kg Cu having higher bone Mg concentrations than those fed diets containing 0, 60 or 80 mg/kg Cu. Data indicated that liver Cu accumulation in sheep depleted of Cu for 3 weeks may be a useful bioassay criterion for determining Cu

bioavailability and that a 10 day bioassay was optimum. However, it is not known whether it was the depletion technique, the higher dietary Cu levels or both that were responsible for Cu accumulation in liver.

Experiment 6 was conducted to determine whether the assay used in Experiment 5 could be used successfully to determine Cu bioavailability from inorganic sources fed to sheep. Sheep were depleted of Cu as in Experiment 5, liver biopsied to determine initial Cu concentrations, then supplemented for 10 days with 0, 20, 40, 60 or 80 mg/kg Cu as reagent grade acetate or 60 mg/kg Cu as feed grade oxide, sulfate or carbonate. The assay was incapable of differentiating among the sources evaluated; however, surgery trauma during biopsy may have confounded the experiment.

Studies reported in this dissertation indicated that liver was the only tissue in which Cu accumulation occurred consistently. The failure of other tissues to accumulate Cu suggests that Cu is under strict homeostatic control in the body. Copper accumulation in liver of chicks provided a useful bioassay criterion for determining Cu bioavailability from inorganic sources. Studies with sheep were inconclusive, and may well reflect the fact that unlike day-old chicks Cu concentrations in liver of sheep are extremely variable. Large differences in initial Cu concentrations may well confound an assay based on Cu accumulation since final Cu concentrations are dependant on initial concentrations, especially during short periods

of supplementation. It is recommended that the bioassay technique be further investigated and that initial liver Cu concentrations be determined in all future experiments. It is also recommended that puncture biopsies be utilized in future experiments since the laparotomy technique appears to severely stress animals.

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BIOGRAPHICAL SKETCH

David Randolph Ledoux was born in Georgetown, Guyana, on April 20, 1952. He attended St. Stanislaus High School in Georgetown and completed his high school education in 1967. The following year he entered the Guyana School of Agriculture and was awarded the Diploma of Agriculture in May, 1970. Following graduation he worked as an Extension Agent for the Guyana Department of Agriculture from 1970 to 1973. From 1973 to 1974 he worked as an instructor at the Guyana School of Agriculture and from 1976 to 1977 he worked as a credit analyst at the Guyana Agricultural Bank.

In August, 1977 the author and his family emigrated to the United States. He entered the University of Missouri in January, 1978 and was awarded the degree of Bachelor of Science in May, 1981. In August, 1981 he was granted a research assistantship and began graduate studies in the area of animal nutrition at the University of Missouri. He was awarded the degree of Master of Science in animal science in May, 1984.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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